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Pathogenicity of Rous Sarcoma Virus for Rats and Rabbits [1,2]

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SUMMARY

Inoculation of Rous sarcoma virus into rat embryos or newborn rats caused a hemorrhagic disease characterized by formation of cysts which filled with serous transudate and then became hemorrhagic. Subsequently, sarcomas appeared in some rats. In newborn rabbits the inoculation brought about a disease characterized by development of mostly benign multiple fibrous nodules from which the virus could sometimes be isolated by irradiation with X or ultra-violet rays. The virus neutralized with the specific antiserum did not cause any pathological symptoms in rats or rabbits. Serums of affected rats did not contain virus antibodies, while those of affected rabbits sometimes contained them. Hemorrhagic disease of rats and fibromatosis of rabbits are interesting models for the study of the interrelation between virus and cell.—J. Nat. Cancer Inst. 26: 1295-1309, 1961.

THE PATHOGENICITY range of tumor viruses has long attracted the attention of many investigators, and it has been claimed that tumor viruses are distinguished by strict species specificity. This is highly characteristic of some viruses such as the Shope rabbit papilloma. Other tumor viruses may affect various species: The Rous sarcoma virus (RSV), while undergoing some changes in its properties (*1*, *2*), can induce tumors not only in chicks but also in ducks, pheasants, and pigeons. Among the viruses known at the present time, SE polyoma exhibits the widest range of oncogenicity in affecting four animal species and causing primary tumors in many different organs that may be distinctly different in cell type. However, in all such cases the oncogenic range never passes beyond the limits of an animal class.

Two questions arise: 1) Can a tumor virus that induces tumors in one class of animals induce tumors or other pathological processes in the representatives of another? 2) What are the efforts necessary to overcome their natural resistance to this virus? These questions were considered in our laboratory for their potential contribution to our knowledge of the pathways of viral tumor propagation. We attempted

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to induce tumors in rats and rabbits by means of RSV (*3–8*). The present paper is a brief summary of the results obtained.

HEMORRHAGIC DISEASE IN RATS AND FIBROMATOSIS IN RABBITS

Rats and rabbits are resistant to RSV; therefore, we tried to produce artificial tolerance to the virus by administering it to embryos and newborns. The supernatant obtained after centrifugation of the chick sarcoma tissue, triturated thoroughly with physiological saline, was injected into rat embryos, on their 16th to 18th day of gestation, through the uterine wall and embryonic envelopes after dissection of the abdominal wall. One week after birth the animals received the virus again under the skin, sometimes repeatedly. Subcutaneous cysts, containing at first a serous and then a hemorrhagic transudate, appeared in rats most frequently 2 to 3 weeks after the last injection (fig. 1). Autopsy of these rats revealed similar small cysts in other loci of the body as well as punctate and diffuse hemorrhages in various tissues and organs, particularly in the pleura, pericardium, and lungs. The lungs of one rat resembled a cluster of vesicles filled with a sterile hemorrhagic transudate (fig. 2).

Histologic examination of the affected tissues revealed: (*a*) an increase in porosity of the capillaries and disruption of their walls, which caused mass diapedesis of erythrocytes (fig. 3), (*b*) loosening of collagen, and (*c*) a large number of mast cells devoid of basophilic granules. The disease noted in the rats was in many respects similar to that of chick embryos infected with RSV (*9*) and therefore called hemorrhagic rat disease. Svet-Moldavsky (*10–12*) observed a similar disease and sarcomas, which appeared in rats after the transplantation RS tissue into embryos and newborns.

In our laboratory among 151 rats treated with RSV (without serum) and long kept under observation, tumors appeared in 8—1 in the thoracic cavity, 3 in the groin, 1 in the axillary space, and 3 at the site of initial administration. Histologically, all these tumors similarly represented spindle-like sarcomas.

The attempt to isolate the virus from these tumors through inoculation to chick and chick embryos gave negative results; however, the study of these tumors is being continued. Svoboda (*13*) succeeded in transplanting such tumors to chicks by irradiating the tumor cells with X rays.

Similar experiments were carried out on rabbits (*4, 6*). From the 1st day after birth, they were treated subcutaneously 4 times every other day with 1 ml of the supernatant, obtained by triturating the Rous sarcoma with a salt solution at a ratio of 1:4. Some of the rabbits were treated with the uncentrifuged fluid.

About 2 to 3 weeks after the last injection, numerous subcutaneous fibrous nodules appeared (fig. 4), which increased in number and volume and sometimes proved lethal. The affected rabbits lagged in growth.

The most severe form of the disease was noted in young rabbits treated with the uncentrifuged sarcoma extract. Autopsy of the succumbed animals showed in some cases fibrous nodes in the liver. Eventually, punctate hemorrhages were also seen in various organs.

Histologic examination in these experiments revealed the following: (*a*) in the nodes, fibrous tissue with a great number of cells, some with degenerating nuclei and others of the fibroblastic series with greatly enlarged nucleoli (fig. 5), (*b*) an accumulation of lymphoid cells around the vessels of the liver and the diaphragm (fig. 6), (*c*) a tendency toward localization of the lymphoid elements around the vessels in other organs and in the subcutaneous connective tissue, and (*d*) release of appreciable numbers of erythrocytes into the perivascular tissue. In surviving rabbits the fibrous nodes had acquired chondral consistency and underwent resorption. Thus in newborn rabbits, RVS was shown to induce fibromatosis accompanied by vascular affections.

Individual strains of RSV differed in their capacity to cause the hemorrhagic disease in rats and fibromatosis in rabbits. Of the 4 strains studied—from Bryan, Carr, Engelbreth-Holm, and Djadkova—Carr's strain was the most active and Djadkova's the least active.[3]

ROLE OF TOLERANCE, DOSAGE, AND REPEATED ADMINISTRATION OF RSV IN REPRODUCTION OF HEMORRHAGIC DISEASE AND FIBROMATOSIS

Initially, we reproduced the hemorrhagic disease by repeated administration of RSV, on the assumption that the first embryonic administration would create tolerance to the virus. In heterotransplantation of RS tissue, Svet-Moldavsky (*10*) reported that the hemorrhagic disease was noted after a single injection of the sarcoma-cell suspension to newborn rats.

Similar results were reported by Svoboda and Grozdanovič (*14*), who observed development of the hemorrhagic disease after injection of minced RSV tissue into the abdominal cavity of newborn rats. Experiments in which the hemorrhagic disease was caused by a single administration of sarcomatous cells presented questions of the role of immunological tolerance in reproduction of hemorrhagic disease in rats, dosage, and multiplicity of administration of the sarcomatous material. These problems have been studied in both this laboratory and that of Dr. M. Hašek in Prague.

Kryukova (*5*) inoculated rat embryos with a single dose of the supernatant obtained after centrifugation of minced sarcoma cells and the uncentrifuged cell suspension. With both materials hemorrhagic disease was recorded, the incidence being higher with uncentrifuged suspension. It was reported by Svoboda and Grozdanovič (*15*) that the incidence of

[3] These strains were kindly given to us by Dr. A. Djadkova, Prof. H. Morgan, and Dr. J. Svoboda.

the disease in rats was higher when induced by 5 postembryonic injections of the virus than by 1 embryonic and 3 postembryonic. There was no increased susceptibility to RSV in rats treated with chick blood or spleen, either in embryonic or postembryonic life or both.

These workers have therefore negated the significance of tolerance for the reproduction of hemorrhagic disease. It seems possible, however, that a single injection of the virus induces tolerance to itself; therefore it survives within the organism up to the moment when this organism already tolerant begins to react to it. Hence induction of the disease by a single dose of virus is not conclusive enough to refute the significance of artificial tolerance to this virus, especially since induction of tolerance depends also on the dosage. The dose and number of injections are important factors in the reproduction of hemorrhagic disease in rats and of fibromatosis in rabbits, whether tolerance is induced or not.

This was demonstrated by Kryukova (*7*), who treated 1- to 2-day-old rats with a single dose of the titrated material. The results obtained are summarized in table 1. The virus material which is active at 1:200 and 1:400 dilution causes the hemorrhagic disease much less frequently than one active at 2000 to 7000 time dilution.

TABLE 1.—Development of hemorrhagic disease in rats after a single inoculation of different doses of RSV

Experiment No.	Virus titer of material inoculated	Time of inoculation	Number of rats	Number of affected rats	Beginning of hemorrhagic disease (days)
1	1 : 200	1st Day after birth	5	1	23
2	1 : 400	"	10	6	10
3	1 : 2000	"	8	8	10
4	1 : 3500	"	3	3	15
5	1 : 3500	"	8	1	30
6	1 : 6000	Into embryo	5	5	5
7	1 : 7000	1st Day after birth	4	4	20
8	1 : 200	2d Day after birth	5	2	25
9	1 : 200	"	5	1	28
10	1 : 400	"	8	3	15
11	1 : 400	"	2	1	30
12	1 : 2000	"	4	4	11

The significance of the total dose of the virus upon multiple administration to rats has also been demonstrated by Morgunova (*8*), who treated newborn rats with the supernatant obtained after centrifugation of chick-sarcoma tissue thoroughly triturated with sand. The titer of the virus was assayed by intradermal titration on chicks. The first injection was made within the first 24 hours after birth and the subsequent ones every day for 3 to 6 days. The results (table 2) show that the quantity of the injected virus is of importance for the reproduction of the hemorrhagic disease and that with sufficient dosage all rats contract the disease; its gravity increases with dosage and a decrease of the incubation period.

TABLE 2.—Development of hemorrhagic disease in rats after multiple subcutaneous inoculations of RSV (virus titer of the material inoculated 1 : 10,000)

Experiment No.	Time of inoculation after birth (days)	Number of injections	Total dose (ml)	Number of rats inoculated	Number of affected rats	Time of appearance of cysts (days)	Type of cysts
1	1	1	0. 2	9	1	150	Single
2	1–3	3	0. 6	3	2	40–80	Single
3	1–3	3	0. 6	7	3	26–34–55	Single
4	1–5	5	1. 5	6	6	15–18–24	Multiple
5	1–6	6	1. 9	3	3	14–18	Multiple

ISOLATION OF VIRUS FROM TISSUES OF AFFECTED RATS AND RABBITS

All our attempts to isolate the virus from diverse materials of affected rats and rabbits and to pass the disease by the administration of this material to rat embryos and newborn rabbits proved futile. In these experiments (*5*) internal organs and the walls of cysts from every affected rat or fibrous nodes of rabbits were triturated with sand and saline (1 : 3), and the suspension was injected into rat embryos or the pectoral muscles of hens or chicks. The material was taken from rats at various stages of the disease. The results are summarized in tables 3 and 4. Negative results have also been obtained in the attempt to isolate the virus by inoculation of chick embryos with this material.

TABLE 3.—Inoculation of material from rats with early symptoms of hemorrhagic disease to chickens (2 chickens inoculated in each experiment)

Experiment No.	Time of inoculation	Dose of suspension (ml)	Age of rats (days)	Material inoculated	Results
1	Embryo	0. 075	4	Internal organs	Negative
2	Embryo, after birth	0. 075 0. 1	10	Internal organs, cyst wall	Doubtful
3	After birth	0. 2; 0. 2	13	Internal organs, cyst wall	Negative
4	Embryo	0. 075	19	Internal organs, cyst wall	Negative
5	Embryo	0. 1	5	Internal organs	Negative
6	Embryo, after birth	0. 15 0. 2; 0. 2	26	Internal organs, cyst wall	Negative
7	Embryo, after birth	0. 05 0. 2	5	Internal organs	Negative
8	Embryo, after birth	0. 15 0. 2; 0. 2; 0. 4	20	Cyst walls	Negative
9	Embryo, after birth	0. 2 0. 2	12	Cysts walls	Negative
10	Embryo, after birth	0. 2 0. 2; 0. 3	10	Internal organs, cyst wall	Negative
11	Embryo, after birth	0. 1 0. 2; 0. 2	17	Internal organs	Negative

TABLE 4.—Inoculation of material from rats in late stage of hemorrhagic disease to chickens (inoculation into embryo and after birth, 2 chickens inoculated in each experiment)

Experiment No.	Suspension dose (ml)	Age of rats (months)	Material inoculated	Method of inoculation	Results
1	0.06 0.2; 0.3	4½	Transudate	Intracutaneously	Negative
2	0.6 0.2; 0.3	5	Internal organs, cyst wall	Intramuscularly	Negative
3	0.1 0.2; 0.2; 0.4	1½	Internal organs	Intramuscularly	Negative
4	0.06 0.2	2½	Lung, cyst wall	Intramuscularly	Negative
5	0.06 0.02	1½	Transudate	Intracutaneously	Negative
6	0.06 0.2	2½	Transudate, cyst wall	Intramuscularly	Negative
7	0.06 0.2; 0.4	3½	Lung, cyst wall	Intramuscularly	Negative
8	0.06 0.2	1½	Lung	Intracutaneously	Negative
9	0.06	3½	Internal organs	Intracutaneously	Negative

Other authors (*10*, *12–14*) sometimes succeeded in isolating the virus in affected rats. But in their experiments the rats were inoculated with very large doses of sarcoma cells, and these conditions could be favorable for the persistence and multiplying of the virus in the organism. Negative results were also obtained in numerous attempts to isolate the virus from fibrous nodes by inoculation of chick embryos. Neither was it possible to reproduce hemorrhagic disease in rats by the material taken from affected rabbits.

Thus it appears that in the majority of cases the active virus cannot be isolated from the organism of affected rats and rabbits. This is no surprise, however, since chick sarcomas inoculated into turkeys do not contain the active virus. In connection with the experiments by Svoboda (*13*) we have attempted to reveal the virus in the material taken from affected rabbits and rats by exposing it to radiation. For this purpose extracts from sand-triturated tissues of affected rats were irradiated with X and ultraviolet rays, then administered to chicks. In other cases the material was first irradiated and then triturated. In controls it was administered before irradiation.

Positive results were obtained in 2 of 8 experiments. In one of these the liver of a hemorrhagic 4½-month-old rat had been previously irradiated with 5000 r and then triturated. The supernatant induced a tumor which was soon resorbed. In the other experiment the walls of cysts and the livers taken from 2 hemorrhagic 5-month-old and 7-day-old rats (triturated and then irradiated) induced tumor growth in chicks. No tumor growth was noted in control experiments with unirradiated material in either experiment.

Irradiated material of affected rabbits gave similar results. Positive

results were obtained in 4 of 8 experiments. In the first, the chick was treated with the minced and then with the ultraviolet-irradiated (2537 A, 10 min at a distance of 50 cm) fibrous node taken from a 26-day-old rabbit. However, the tumor thus formed could not be subcultured via the filtrate.

In the second experiment administration of the fibrous node, taken from a 1-month-old rabbit and irradiated for 5 minutes with ultraviolet at a distance of 10 cm in one experiment and for 10 minutes at 40 cm in another, induced sarcomas in all 4 chicks. The mucous and hemorrhagic tumors were successfully subcultured.

In the third experiment, a node from a 45-day-old rabbit, irradiated (5000 r) and then triturated, was administered to 3 chicks. In each of these, typical sarcomatous tumors appeared, which were transplantable through the filtrate. The titer of the virus in one of these tumors amounted to 1:10,000.

Finally, in the fourth experiment, we used a 23-day-old rabbit node which was minced and then irradiated (5000 r). When administered to 2 chicks it induced typical sarcomas in both. The tumors appeared in no control experiment in which the same but not irradiated rabbit material was administered to chicks.

It may be suggested, then, that in rats the virus is masked by some inhibitor which can be partly destroyed by ionizing radiation. The respective experiments are still continued.

NEUTRALIZATION EXPERIMENTS

The following procedure was used in experiments in which rats were treated with RSV neutralized by a specific immune serum (*7*). One litter was divided into two parts. One of these was inoculated with RSV, strain Carr. After centrifugation of the sarcoma tissue triturated with physiological saline in the dilution of 1:10 or 1:15, the supernatant was mixed with an equal volume of immune serum. The other part of the litter was inoculated with the same proportions of RSV preparation and normal rabbit serum. After a 45-minute incubation period at 37° C they were injected intramuscularly into chicks. The results obtained are summarized in table 5, which indicates that the virus neutralized by the specific serum does not induce any hemorrhagic disease. The serum of rabbits immunized with the Djadkova strain, which did not cause hemorrhagic disease, neutralized the Carr and Engelbreth-Holm strains, which did not cause the disease in young rats when mixed with this serum.

Recent experiments (*16*) also showed that RSV neutralized by a specific serum can no longer induce the hemorrhagic disease in young rats. The effect of the specific immune serum on the fibromatosis-inducing virus of rabbits has also been studied (*6*).

A virus containing supernatant (dilution 1 : 5) was mixed with an equal volume of undiluted immune serum and the mixture incubated for 45

TABLE 5.—Infection of rats, first 2 days after birth, with RSV (Carr's strain) neutralized by rabbit immune serums*

Experiment No.	Number of rats	Inoculation with:	Results
1	4	RSV 1:10	Hemorrhagic disease in all rats
	7	RSV 1:5 mixed with equal dose of immune serum	Remained healthy
2	3	RSV 1:10	Hemorrhagic disease in all rats
	3	RSV 1:5 mixed with equal dose of immune serum	Remained healthy
3	5	RSV 1:5 mixed with equal dose of normal rabbit serum	Two rats became ill
	5	RSV 1:10	Two rats became ill

*Serums of rabbits immunized with the Carr strain of RSV.

minutes at 37° C, whereupon it was administered to rabbits within their first days of life. We gave the mixture also to hens to ascertain whether the virus was completely neutralized. Half the young rabbits treated with this mixture were inoculated with the same extract, which was mixed under similar conditions with normal rabbit serum. Serums immune to the Carr and Djadkova strains were tested.

Results in table 6 show that the virus neutralized with immune serum is deprived of its ability to cause fibromatosis. In some experiments the neutralized virus caused the appearance of single nodules (because of incomplete neutralization) which underwent rapid resorption, but in control animals lethal fibromatosis did develop.

TABLE 6.—Inoculation of young rabbits with RSV (Carr's strain) neutralized by rabbit immune serums

Experiment No.	Number of rabbits	Infection with:	Results
1	4	RSV mixed with anti-RSV (Carr) serum	One rabbit developed nodule, regressed in short time
	4	RSV mixed with normal rabbit serum	Multiple fibromatosis in all rabbits
2	3	RSV mixed with sarcoma virus (Djadkova strain) anti-serum	Remained healthy
	4	RSV mixed with normal rabbit serum	Multiple fibromatosis in all rabbits
3	4	RSV mixed with sarcoma virus (Djadkova strain) antiserum	Two rabbits remained healthy*
	3	RSV mixed with normal rabbit serum	Multiple fibromatosis in all rabbits
4	2	RSV mixed with sarcoma virus (Djadkova strain) antiserum	One rabbit remained healthy; the other developed small nodule
	3	RSV mixed with normal rabbit serum	Mortal fibromatosis in all rabbits
5	6	RSV alone	Mortal fibromatosis in all rabbits

*Two of the 4 developed 3 small nodules and regressed in a short time.

The results obtained with the virus neutralized by the specific serum show conclusively that the hemorrhagic disease of rats and rabbit fibromatosis are actually caused by RSV.

SEROLOGIC STUDIES

Nartsissov, Biryulina, and Kryukova (*17*, *18*) have considered these questions: 1) Do serums of hemorrhagic rats contain complement-fixing antibodies against extracts of sarcoma and normal chick tissues? and 2) Do the cysts contain antigens which react with serums of sarcoma-immunized animals? Their results showed that none of the 10 serums of rats treated with the virus in the embryonic and postnatal stage reacted with RSV or normal muscle extracts. Rats treated with RSV but not affected with the hemorrhagic disease (14 serums) also gave negative results. Immunization of the affected and unaffected 2- to 3-month-old rats with the sarcoma suspension was accompanied in half of the animals (14+ and 13−) by formation of antibodies to the virus and chick antigen. Negative results were also obtained in the tests of hemorrhagic rats for antibodies which neutralize RSV.

Serum of sarcoma-immunized rats eventually reacted to the extract from the walls of the cysts. However, serums of rats immunized with normal chick organs gave similar results. These reactions might possibly be due to heteroantigens which are common to chicks and rats; this problem requires further inquiry.

Somewhat different results were obtained in experiments with the serums of fibromatosis-affected rabbits. About half the serums gave positive results of complement-fixation test with sarcoma extract (8+ and 11−). After additional immunization with sarcomatous material, the affected rabbits also produced antibodies to sarcomatous and normal chick tissues. Lack of tolerance to antigens of the sarcomatous and normal chick tissue is presumably accounted for by the fact that only a small dose of these antigens was initially given to rats and rabbits.

The fibrous nodes were also tested for the viral and chick antigen by means of the complement-fixation reaction with serums of rabbits immunized with sarcomatous material. These experiments showed that extracts of fibrous nodes do not react with antiserums to sarcoma and normal chick tissue. Thus the complement-fixation reaction does not reveal either viral or the chick component in the fibrous nodes.

DISCUSSION

The pathogenic potencies of RSV have often been studied by numerous workers, whose results showed that its properties usually change upon adaptation to certain tissues of chicks and other birds (*1*, *2*, *19*). Our experiments show that the virus can be pathogenic to mammals.

In 1957, almost simultaneously with publication of our first data on the hemorrhagic disease of rats, Hamazaki *et al.* (*20*) reported on the

pathogenicity of RSV in guinea pigs, mice, and hamsters. These workers transplanted RSV into the brains of these animals, where an agent was found which caused inflammatory phenomena, but no tumors. This agent was neutralized with RSV antiserum, but did not induce any tumors in chicks.

Neutralization of this agent by the antivirus serum was tested in mice in which it caused pneumonia, diagnosed only histologically. No data will be found in this publication to judge the reliability of the test (particularly on the constancy of the effects in mice). Additional inquiry seems necessary, therefore, before one can conclude that the agent isolated from mice and other animals is actually an altered RSV.

The evidence obtained in our laboratory demonstrates the wide pathogenic potencies inherent in RSV and that by means of this virus, which under natural conditions affects only birds, it is possible to inoculate animals of another class as well, *e.g.*, mammals. This virus, which induces malignant metastasizing tumors in chicks, under the conditions defined, produces hemorrhagic disease in rats (an infectious type of effect devoid of any neoplastic character), but only in the course of time does it induce tumors and a benign neoplastic process in rabbits. Except for some cases, the virus cannot be isolated from the tissues affected. Irradiation experiments suggest that the virus is probably fixed by an inhibitor, which is destroyed by irradiation. It might also be assumed that irradiation breaks the bonds between the virus and the protein. Blocking of the virus by extracts of unaffected tissues of affected rats proved futile. The inhibitor is probably accumulated only in the tissues affected. A study is now in progress to obtain an answer to this question.

The hemorrhagic disease in rats and the tumors which appear afterward, as well as fibromatosis elicited in rabbits by the RSV, are new models whose study may prove fruitful for the elucidation of the nature and mechanism of the neoplastic process. For the first time it is possible to follow in the same animal the infectious and neoplastic process caused by the same virus.

The mechanisms of virus action in hemorrhagic disease and of tumor formation in rats are no doubt different, and a comparative study of both seems to be of primary importance. In the hemorrhagic disease we are apparently dealing with a direct effect of the virus on the vessels of the animal which results in their destruction, whereas the changes of tumor formation in rats and rabbits are of quite another neoplastic character and connected with another tissue. It seems worthwhile to explore the conditions under which the virus acts within the same organism, first as a destructive agent of the conventional infectious type and then as a neoplastic virus.

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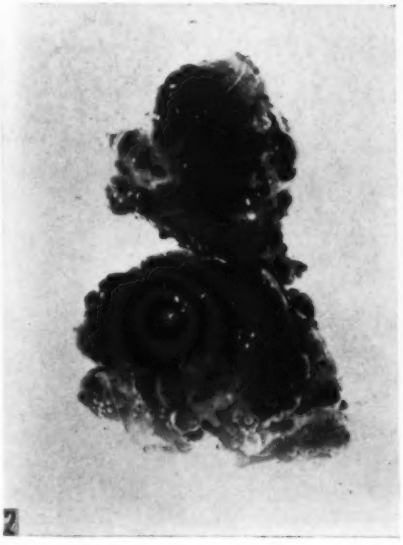
PLATE 196

FIGURE 1.—Subcutaneous cyst of rat with hemorrhagic disease.

FIGURE 2.—Lung of rat with hemorrhagic disease.

FIGURE 3.—Hemorrhages in lung of diseased rat. × 208



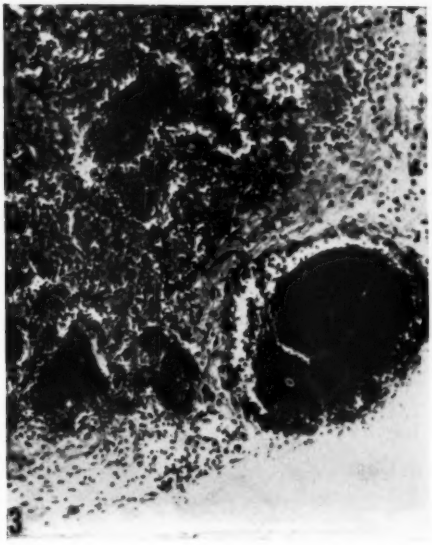


PLATE 197

FIGURE 4.—Fibrous subcutaneous nodes developed in rabbits after introduction of RSV.

FIGURE 5.—Fibrous node of rabbit. Cells with enlarged nucleoli. × 665

FIGURE 6.—Accumulation of lymphoid cells around blood vessels of diaphragm. × 345

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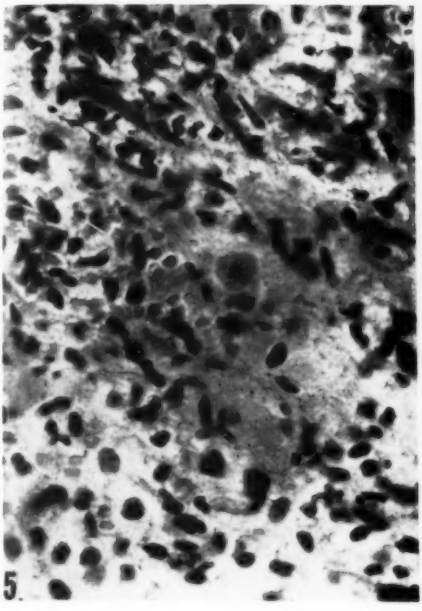
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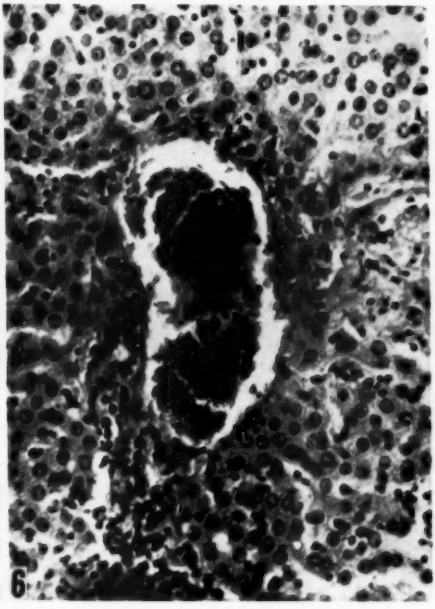
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On the Interaction Between Tumor Viruses and Cells: A Virogenetic Concept of Tumorigenesis [1, 2]

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SUMMARY

This paper presents a virogenetic concept of tumor origin. A virus induces a hereditary transformation of a normal into a tumor cell, conditioned by the supplementary genetic information brought into the normal cell with the nucleic acid of the virus. Such a process cannot be considered a mutation, which is a change of the appropriate genome of the cell, but as a conversion of a normal into a tumor cell which involves incorporation of a genetic substance carrying additional genetic information. The reproduction of the cell with transformed heredity leads to the formation of a tumor, and as an entity the virus will take no part in this process, though it may still exist in the mature form in some tumors. Certain alterations of antigenic structure occur in tumor cells, and they cease then to submit to the growth regulation systems of the organism. The data presented show that some tumors induced by chemical or physical factors are conditioned by latent viruses; therefore the above concept may have a general significance.— **J. Nat. Cancer Inst. 26: 1311–1319, 1961.**

THE INTERACTION of tumor viruses with animal cells is a problem which has long occupied many investigators. Recently it was discussed in detail at a symposium sponsored by the American Cancer Society, Inc., on the possible role of viruses in cancer (*1*). The interest in this problem is comprehensible, since any further inquiry into the nature of tumors, including human tumors, depends on the interpretation of the available evidence. In my view, the elucidation of the role of the virus at individual stages of carcinogenesis and the mode of its action upon the cells are of major importance for the problem of interaction of tumor viruses with cells. It is this problem which I wish to discuss.

ON THE ROLE OF VIRUSES AT INDIVIDUAL STAGES OF CARCINOGENESIS

The intricate process of carcinogenesis can be divided into several stages, the principal ones being conversion of a normal cell into a tumor

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cell and reproduction of the tumor cells thus formed. The tumor virus carries out this conversion. Is the active virus necessary, however, for the subsequent stage of carcinogenesis, the reproduction of these cells for tumor growth? Some evidence suggests that it is not.

A small amount of Rous virus administered to chicks cannot be revealed in the mature tumors (*2*) or in old chick tumors induced by larger amounts of the virus, though they continue growing (*3*). Neither can Rous virus be isolated from fibromatous nodules induced by the virus in newborn rabbits (*4*). The Shope papilloma virus cannot be extracted from vigorously growing papilloma of domestic rabbits (*5*), or SE polyoma from hamster tumors induced by this virus (*6*).

It may be inferred from these as well as similar observations that the role of the virus in tumorigenesis is different from that in tumor growth. Although a viral tumor cannot arise without a definite dose of the virus, it can presumably continue growing despite the drop of viral concentration in the tumor or even after complete disappearance of the active virus.

Two questions arise: 1) What is the fate of the tumor-inducing virus when it cannot be revealed in the growing tumor? and 2) Why does a tumor grow, though devoid of detectable virus? Several explanations may account for these phenomena. It might be assumed that the tumor cell is a less favorable medium than the normal cell for reproduction of the respective virus, and that virus inhibitors appearing in the tumor cell cause the virus to reproduce less vigorously. The virus may cause some genetic change in the tumor cell so that it does not require more virus for further reproduction. Some evidence indicates that the virus is present in the tumor in a masked form, as Rous virus in pigeon tumors; sometimes only the virus antigen can be revealed, as is true with the Shope carcinoma. It appears from this and other evidence that a viral tumor continues growing, though the virus has lost its carcinogenic properties. Hence, the carcinogenic ability of a virus and a tumor-inducing carcinogen would not affect tumor growth or its motive force (*cf. 7*).

ON THE ALLEGED PARTICIPATION OF VIRUSES IN THE INDUCTION OF TUMORS BY CARCINOGENIC SUBSTANCES AND TREATMENT

It has been suggested that carcinogenic compounds activate latent tumor viruses or provide optimum conditions for their reproduction (*8*). The following experiments had been carried out in this laboratory as early as 1945 to test this hypothesis (*9*, *10*).

Tumors were induced in mice by dibenz[*a*,*h*]anthracene. Very early, just palpable nodules were triturated in the cold, with physiological saline under conditions preventing oxidation, and filtered through a Berkefeld V candle. To secure best conditions for reproduction of the viral agent presumed to be present in these filtrates, we administered them to mice treated with very small doses of dibenz[*a*,*h*]anthracene that did not induce tumors

in control animals. The filtrates were administered at the same site as the carcinogen. Of 114 mice treated in this way, tumors appeared in 18. Of 67 mice treated with the carcinogen alone, only one appeared. The results were always negative in similar experiments with filtrates from mature tumors.

This experiment indicates that in very young tumors induced by a carcinogen, a virus-like agent can eventually be detected which causes the formation of the tumor but is subsequently masked or eliminated from the growing tumor. The agent apparently resided in the nucleus, since the filtrates of isolated and minced nuclei eventually proved carcinogenetic in some experiments. The carcinogen apparently activates a latent virus already residing in the animal. Unfortunately, we did not dispose of inbred animals at that time, nor were newborn animals used. Yet the difference between the experimental and control groups was distinct.

Švec *et al.* (*11*) showed that a transplantable rat tumor contains a latent viral agent which induces myeloid leukemia in newborns. Some recent publications emphasize a connection between radiation-induced tumors and latent viruses. Gross (*12*, *13*) induced leukoses in mice of a nonleukemic strain (C3H) by exposing them to X rays. When administered to newborns, filtrates of the affected mice induced leukoses in 11 percent of mice of the same strain.

Similar data have been obtained concerning mice of the C57BL strain (*14*). Carcinogenesis induced by other factors also may be linked with latent viruses. Mazurenko and Nadgornaya (*15*) induced leukoses in mice by means of the vaccinia virus. For this purpose 3- to 20-day-old mice of the CC57BR strain (*16*) received subcutaneous injections of a single dose of the vaccinia virus cultured on the chorio-allantois of a chick embryo. Of 115 mice surviving the 7th month, 33.6 percent had leukoses within 12 months. In the 132 control animals of the same strain, no leukemia had been noted. In the affected mice various forms of leukoses were found, *e.g.*, myelosis, hemocytoblastosis, and aleukemic reticulosis. In some cases subcultures were successful. Similar experiments on noninbred albino mice proved negative.

It was shown subsequently by Mazurenko (*17*) that leukosis induced by the vaccinia virus can be passed through filters and that the mechanism of leukosis induction likewise involves a latent virus. It is interesting to compare these data with those of Duran-Reynals (*18*), that vaccinia virus can induce tumors in mice whose skin has been painted with methylcholanthrene and which have received cortisone and estrogen. The vaccinia virus could not be isolated from the tumors thus formed, and in control mice no tumors developed.

Thus carcinogenic agents, such as certain chemicals, irradiation, and infectious virus, may not affect the cell directly; the carcinogenesis may be an indirect effect via the latent virus already present in the organisms.

It would naturally be unwise to conclude that carcinogenesis caused by physical and chemical agents is always so affected. It may, however, be postulated that the alleged participation of viruses in carcinogenesis

caused by these agents is based on experimental evidence. Moreover, those who claim that carcinogenesis is induced directly by carcinogens may be requested to present evidence that in their experiments any effect of latent viruses has been excluded.

Whatever the results of further studies on the role of viruses in tumors induced by chemical or physical agents, it will be true that the tumor-inducing factor is not significant for further tumor growth. The reason for this may be that the conversion of a normal into a tumor cell is genetically conditioned by a change in the cell genome.

ON THE CHARACTER OF HEREDITY CHANGES OF THE TUMOR CELL

It has been suggested that the conversion of a normal into a tumor cell is due to somatic mutation (*19*). Can we accept this hypothesis? The experiments (*9*, *10*), in which a virus-like agent could be detected at an early stage of formation of carcinogen-induced tumors but not at maturity, led to the implication that the virus causes hereditary changes in the tumor cell. This hypothesis is also supported by some evidence on genetic changes of bacterial cells available at that time. In 1923 I described some hereditary changes of the serological types of *Proteus vulgaris* (*20*). Later such transformation was described in the pneumococci group (*21*), and it was shown that the transforming substance of pneumococci is deoxyribonucleic acid (DNA) (*22*). The virus of rabbit fibroma was transformed into a myxomatosis virus, and according to present data the transforming agent was a nucleoprotein (*23*, *24*).

These data are connected to the alleged role of viruses in genetic changes of tumor cell (*25*, *26*). It has been postulated that the virus affects the cell genome by transforming the normal cell into a tumor cell but not participating in any further stage of carcinogenesis. Much more ample evidence is now available to substantiate this hypothesis—first, that pertaining to the system of bacteriophage. These data are mentioned only briefly, since they have already been discussed at length (*1*). In fact, lysogenic conversion has much in common with the tumor-cell-virus system, since in both, the virus causes new characters in the cell and is not reproduced in mature form. The mechanism of lysogeny is known to be linked with the incorporation of the genetic material of the phage (its nucleic acids) into a locus of the bacterial chromosome.

There are some grounds to assume that neoplastic transformation is likewise due to incorporation into the cell of the genetic material of the virus and to the additional genetic information which the cell thereby receives. Of importance in this connection are the data of Harel and Lacour *et al.* (*27*, *28*) according to which ribonucleic acid (RNA)-containing preparations from leukemic tissues of man and mice cause tumors in mice. The data of Latarjet (*29*, *30*) show that nucleic acid of leukemic tissues of AK mice accelerates the appearance of leukoses in mice of the same strain and induces diverse tumors.

The fact that RNA isolated in leukoses is oncogenic may suggest that in tumor viruses nucleic acid is likewise a genetic substance modifying the synthetic processes in the cell. Direct evidence of the ability of DNA isolated from the polyoma virus to transform a normal cell into a tumor cell has recently been brought forward by DiMayorca *et al.* (*31*). Thus nucleic acids of tumor viruses may act as a source of cell information which is necessary to convert a normal into a tumor cell.

Thus conversion of a normal cell into a tumor cell may not be a mutation in the conventional meaning of the word. Mutation is a change of the appropriate genome of the cell, whereas conversion of a cell into a tumor cell (like lysogenation) involves incorporation of a genetic substance carrying additional genetic information into the cell and in some way integrating with the cell genome. This is hybridization rather than mutation (*32*). Integration of the genetic substance of the virus may bring about some changes in the cell genome as well, but this change is closely associated with the additional genetic substance of the virus.

If hereditary conversion of a normal into a tumor cell is linked with incorporation into its genome of viral elements, it might be expected that elimination of these elements from the cell would result in its reversal to the normal state. Such a conversion of lysogenic cultures into normal ones has actually been described, *e.g.*, in *Bacillus megatherium*. This process should not be regarded as impossible in the tumor cell.

On this assumption our efforts in cancer therapy should be focused on the eradication of additional genetic information brought into it by the virus rather than on that of the tumor cell itself. In forthcoming years cancer therapy may follow this path. The interrelations between the tumor virus and the cell may adopt various aspects.

In many cases synthesis of mature viral particles takes place as well as incorporation of the viral component into the cell genome. In still other cases the process may be similar to the formation of a lysogenic culture, and the active virus is not detectable in the cell at all. Such a process might possibly take place in the "nonviral" cancer of mammary glands of mice, which was studied by Mühlbock (*33*). A study of carcinogenic properties of nucleic acids isolated from such tumors seems to be worth while.

Always, however, the cells undergo genetic changes which are linked with incorporation into the genome of the nucleic acid of the virus, whether mature viral particles form or not. Subsequently one may state that cancer is a neoplastic conversion of cells caused by molecular infection with the nucleic acid of the tumor virus. Clinical manifestation of this infection depends on numerous factors.

Such a definition can as yet be applied only to tumors of viral etiology. If the direct effect of physical and chemical agents upon the cell genome causes its conversion into a tumor cell, the process would be linked with the appropriate genome of the cell but not with the additional genetic information. In this case one may think of cell mutation in the conventional meaning of the term. However, it is hard to believe that processes

resulting in essentially similar changes of the cell might be induced once by a change in the genetic information of the cell proper, while in other instances by additional genetic information received by the cell from the outside. The necessity of a thorough study of the role of latent viruses in carcinogenesis caused by other factors comes again to the foreground.

VIRUSES AND CONTROL OF CELL REPRODUCTION

Most workers believe that vigorous cell reproduction resulting in tumor formation is caused by the release of tumor cells from the control of the growth-regulating systems. Alov (*34*) has brought forward experimental evidence corroborating this viewpoint. In his experiments, thyroidin, which stimulates mitosis in normal cells, and cortisone, which exerts an antimitotic effect, did not change the mitotic activity of tumor cells when administered in doses that distinctly affect normal cells. Why are tumor cells released from the control of the growth-regulating systems?

A hypothesis has been advanced by the writer (*35*) that the changes in protein synthesis caused by the etiological factor alter the antigenic structure of tumor cells. It is precisely this peculiarity of tumor cells that enables them to reproduce beyond the control of the regulating systems. In the course of evolution a strict correspondence has been established between the protein composition of the cell and the regulating systems of the organism. As soon as this relation is upset the control of cell reproduction is deranged.

Hypotheses linking carcinogenesis with a change in the antigenic cell structure have also been developed by Green (*36*). Does the virus, however, cause any changes in the antigenic structure of tumor cells? Adequate evidence is available to show the presence of new antigens in the cells of the Shope papilloma and Rous sarcoma. If the virus is eliminated from a papilloma extract by adsorption on erythrocytes, the anaphylaxis reaction will reveal in this extract a new antigen lacking in the skin of healthy rabbits (*37*). This is a cellular antigen, not viral.

Such an antigen has also been detected in the supernatant of the Rous sarcoma homogenate after centrifugation at 60,000 $\times$ *g* (*38*). This virus-deficient supernatant fluid gave a distinct precipitation line with the serum against Rous sarcoma absorbed by normal chick tissue. Purified antibodies against sarcoma virus that neutralized this virus did not precipitate the antigen of the supernatant. It appears from these data of our laboratory that, in the viral tumor tissue, antigens can be detected which are lacking in the respective normal cells. It is noteworthy that new antigens also appear upon lysogenation of bacteria (*39*).

From the vast evidence obtained in this as well as in other laboratories it follows that specific soluble antigens can be detected in tumors of various origin and that the change of antigenic structure of tumors occurs in two directions: 1) complication involving the appearance of new antigens, and 2) simplification with disappearance of the antigens appropriate to normal cells (*40*).

It is not surprising that the regulatory systems, which have been turned by evolution to the regulation of reproduction of cells with a definite antigenic structure, are unable to regulate reproduction of cells with another antigenic structure so that they begin to reproduce chaotically. This reproduction is possible only when the immunological response of the organism is not very pronounced. This is actually the case, which is presumably connected with the fact that new antigens do not get into the blood and hence cannot cause any immunization stimulation.

In summary, tumorigenesis may be conceived of as follows: The tumor viruses keep for a long time symbiotic relations with the organism. Numerous factors, such as hormone treatment and ionizing radiation, can derange this symbiosis and provide for conditions which may favor the integration of the nucleic acid of the virus in the cell. Prolonged proliferation of the cells caused by different factors is presumably favorable for such an integration. The cell receiving additional genetic information undergoes some hereditary metabolic changes resulting in proteins of another antigenic structure being synthesized. These, as well as some other changes, such as enzymatic ones, release the cell from subordination to systems which regulate cellular growth, and inadequately controlled cell reproduction results in the formation of a tumor.

This concept is certainly very schematic and allows much space for analogies and hypotheses. It is based on precise facts, though its universal significance is debatable. In view of rapid accumulation of data in modern experimental oncology it seems worth while to attempt some generalization of these facts. Further progress in cancer research will show whether this concept is to be discarded or extended in accordance with the results of forthcoming studies.

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Triadic Labeling with I^{130}, I^{131}, and I^{133} for Controlled Determinations of Tumor-Localizing Antibodies [1, 2, 3]

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SUMMARY

The triadic label technique was developed to provide a method for the comparison of pairs of tumor-localizing antibodies, over and above a variable background localization of nonspecific globulin. The technique involved the labeling of one antiserum globulin with one radioiodine isotope, the other with a second radioiodine isotope, and the control globulin with a third radioiodine isotope. To exemplify the use of the technique, I^{130}, I^{131}, and I^{133} were selected as triadic labels in two experiments. In one experiment the localizing properties of antihepatoma and antiliver antibodies were compared, over and above the background localization of normal globulin, in rats bearing hepatomas induced by *N*-2-fluorenylacetamide. In the second experiment the localizing properties of antihepatoma and antirat-plasma antibodies were compared, over and above the background localization of antiratserum antiserum globulin, in rats bearing induced hepatomas and in normal rats.—J. Nat. Cancer Inst. 26: 1321-1333, 1961.

THE PAIRED label technique permits the direct measurement of tumor-localizing antibodies in individual tumors over and above the nonspecific background localization of control material (*1*). The globulin fraction of an antitumor antiserum is labeled with one radioiodine isotope and the globulin fraction of normal serum or other control protein is labeled with another radioiodine isotope. The two preparations are mixed and injected simultaneously into the tumor-bearing host. Localization of antibody in the tumor specimen is determined by analysis for the percentage of the injected dose of each of the two isotopes present. In essence, the experimental subject is its own control subject. With I^{131} and I^{133} as members of the pair, the technique has been used to solve a number of problems (*2–5*).

The triadic label technique is an extension of the paired label in which the individual localizations of three different proteins, each labeled with

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a different radioiodine isotope, are determined simultaneously in the same subject. In the experiments described the technique was used to analyze the localization properties of pairs of antiserum globulins, over and above a variable nonspecific background localization of control material, in rats bearing tumors induced by *N*-2-fluorenylacetamide. I^{130}, I^{131}, and I^{133} were used as members of the radioiodine triad.

For normal organs of experimental rats, the background localization of nonspecific material can be estimated satisfactorily from the localization of control globulin in other rats used as control subjects (*5*). In very special cases it can also be estimated for the total tumor and liver portions of abnormal tumor-bearing livers (*5*) from the assay data of control material in other rats bearing like tumors. More generally, however, the background localization of nonspecific globulin, because of its wide variation in the total as well as the subdivided tumor and liver portions of tumor-bearing livers, must be determined for each individual experimental subject.

Labeled localizing antibodies, which have been purified and concentrated *in vitro* before injection, can be recognized much more easily than those in whole antiserum globulins in which the bulk of labeled material is nonspecific. In fact, the magnitude of the uptake of highly purified antibodies in tissues is so much greater than that of nonspecific background material that the latter need not be measured. On the other hand, before antiserum globulins are selected for the purification of antibodies, their localization properties as whole globulins should be assessed. As a result, antibodies with interesting localizing properties may, perhaps, be found; futhermore, the elements upon which they localize may then be concentrated and used as media for the purification of the antibodies in question.

The triadic label technique was developed to provide a method for the comparison of localizing antibodies, prior to their purification from whole antiserum globulins.

MATERIALS AND METHODS

Preparation of antiserums against induced hepatomas, liver, plasma, and serum of rats.—Liver-bearing hepatomas, induced by *N*-2-fluorenylacetamide (*5*), were separated from macroscopically normal-appearing portions of the livers. Sediments of the tumor and normal-appearing parts were prepared by repeated centrifugation at 1,500 × *g* from 10 percent homogenates of the tissues in borate-buffered (*p*H 8) 0.9 percent NaCl. The sediments were injected into rabbits intraperitoneally as suspensions of 100 mg lyophilized material suspended in 5 ml borate-buffered (*p*H 8) 0.9 percent NaCl. A series of 9 injections per rabbit was made over a period of 3 weeks followed by a week of rest before exsanguination. At the end of this time 4 rabbits which had received

tumor sediment were exsanguinated and their serums pooled; 4 rabbits which had received liver sediment were also exsanguinated and their serums pooled. The globulins from these pools of antiserums were assayed in experiment 1. Four more rabbits, which had received tumor sediment as above, received an additional 3 injections during the 5th week and were exsanguinated at the end of the 6th week to give the pooled antitumor antiserum used in experiment 2.

Rat plasma (from oxalated blood) and serum were obtained from normal, male Sprague-Dawley rats, 400 g in weight, of the Charles River variety. Four rabbits received intravenous injections of undiluted plasma and 4 others undiluted serum for 3 weeks in a series of 9 injections, 2 ml per injection. At the end of the 4th week the rabbits were exsanguinated; the antiplasma antiserums were pooled in one batch, and the antirat-serum-antiserums pooled in another. Normal serums were obtained from rabbits receiving no injections.

Radioiodination of antiserum globulins.—Globulins were separated from the antiserums according to our version of the Kekwick sodium-sulfate procedure (*6*). All iodinations with I^{131} (obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.) and I^{133} (obtained from Brookhaven National Laboratory, Upton, Long Island, N.Y.) likewise were carried out according to a procedure previously described (*6*); however, because of the unavailability of I^{130} (obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.) as a carrier-free isotope, iodination with it was carried out according to a slightly modified procedure.

In the carrier-free radioiodine, the isotope is added to nonradioactive carrier iodide (0.065 mg) in 1 N HCl and treated with sodium nitrite to liberate iodine. The solution is neutralized, buffered to *p*H 8 with borate, and added to a solution of 20 mg antiserum globulin in borate-buffered (*p*H 8) 0.9 percent NaCl. Free iodide is removed by ion exchange. Final volume is routinely 20 ml; final yield of bound radioactivity, 23 ± 4 percent of the initial radioactivity; final yield of bound iodine, 0.91 ± 0.17 atoms per globulin molecule. With such an iodination procedure there is no danger of losing antibody activity as a result of the chemical reaction. Such loss would occur only when more than 2 atoms of iodine per protein molecule were bound (*7*). Thus up to 2000 mc I^{131} or 15,000 mc I^{133}, without added carrier, could be coupled theoretically to 20 mg of antibody globulin without *chemically* lowering antibody activity.

I^{130} could be obtained commercially only with carrier iodide (as I^{129}) present, and thus the amount of radioactivity that could be coupled to antiserum globulins was limited. For example, the preparation of I^{130} used for experiment 1 had a specific activity of 16 mc/0.065 mg iodide as of the day of shipment, 0.09 mc as of the day of assay 4 days later. The amount of radioactivity (23% of total) coupled to 20 mg of the antiliver antiserum globulin, as of the day of assay, was therefore limited to 0.02 mc.

Preparation of the triadic label for injection.—After each of 3 antiserum globulins was labeled with a member of the radioiodine triad, the 3 labeled

proteins were mixed, and 4.0 ml portions of the mixture were injected into each rat and into a 100 ml volumetric flask. From the flask, filled to the mark with saline, 1 ml samples were obtained to serve as 1 percent dosage standards.

The determination of the triadic label.—In the determination of the relative amounts of each iodine isotope in the presence of the other 2, advantage may be taken of either of 2 bases for measurement: the characteristic half life of each or the characteristic γ-radiation spectrum of each. If the half lives are exploited, each sample must be counted at least 3 times at adequately spaced intervals, 1 day apart for the triad used in these experiments. If the characteristic γ-radiation spectrum is used, each sample is measured only once, but the radiation detected by the NaI(Tl) scintillation head is recorded simultaneously on 3 different scalers, each of which is set up to receive a different portion (channel) of the total energy spectrum. In the trichannel scintillation spectrometer used for our experiments, channel J (background of 30 cpm) counted integrally all detected activity above 0.65 Mev, channel K (background of 8 cpm) counted differentially between 0.56 and 0.50 Mev, and channel L (background of 12 cpm) counted differentially between 0.39 and 0.33 Mev. I^{130} radiation, as detected by our 2-inch NaI(Tl) crystal, revealed no narrow-banded, high-peaked portions in its spectrum; however, as shown in table 1, the J channel could be used as a satisfactory receiver for I^{130} γ-radiation ($T_{½}$ =0.53 days) exclusive of I^{131} and nearly so of I^{133}. The γ-radiation characteristic of I^{131} ($T_{½}$ = 8.1 days) was counted mainly in the L channel. I^{133} radiation was counted mainly in channel K ($T_{½}$ = 0.94 days), but since the Brookhaven preparation also contained I^{131}, there was a nearly equivalent amount of radiation countable in the L channel ($T_{½}$ = 8.1 days) on this 4th day after fission.

Because of the spectral overlaps (table 1), simultaneous equations summing up the count rates in all 3 channels for each individual isotope preparation in the mixture must be solved. The data required for such a solution include not only the count rate of the unknown mixture in the 3 channels, but also the count rates of the individual isotopes as received and before being mixed. However, since only ratios of count rates between channels for the individual isotope preparations are needed, any amount of each isotope can be used up to that causing coincidence pile-up. Typical ratios can be seen in the last 3 columns of table 1. The set of simultaneous equations summing up the count-rate contribution of each isotope preparation in the 3 channels is given below where

X = count rate in all channels due to I^{130}
Y = count rate in all channels due to the I^{133} preparation
Z = count rate in all channels due to I^{131}

J = total count rate in J channel
K = total count rate in K channel
L = total count rate in L channel

A = ratio of count rate of I^{131} in the K channel to that in the L channel
B = " " " " " I^{133} " " " " " " " " " " "
C = " " " " " I^{130} " " " " " " " " " " "
D = " " " " " I^{131} " " J " " " " " " " "
E = " " " " " I^{133} " " " " " " " " " " "
F = " " " " " I^{130} " " " " " " " " " " "

Since $X + Y + Z = J + K + L$, and since, as shown by table 1, $D = O$, it follows that

$$X = \frac{(K-AL)\,E - (B-A)\,J}{(C-A)\,E - F(B-A)}\,(F + C + 1)$$

$$Y = \frac{-(K-AL)F + (C-A)\,J}{(C-A)\,E - F(B-A)}\,(E + B + 1)$$

$$Z = (J + K + L) - (X + Y)$$

TABLE 1.—Distribution of count rates of individual radioiodine isotope preparations, as received, among 3 channels

Channel*	Count rate of radioiodine isotopes (cpm)†			Ratio of each channel count rate to the count rate of channel L		
	131	133	130	131	133	130
J	0	1140	33300	0. 000	0. 035	1. 28
K	1720	20550	27600	0. 054	0. 64	1. 06
L	31600	32200	26000	1.	1.	1.

*Channel J counted integrally above 0.65 Mev; channel K counted differentially between 0.56 and 0.50 Mev; channel L counted differentially between 0.39 and 0.33 Mev.

†Corrected for background count rates of 30 cpm in J channel, 8 cpm in K channel, and 12 cpm in L channel.

In each assay appropriate samples of pure isotopes and standards representing 1 percent of the injected radioactive dose were counted before and after each set of experimental samples. A set contained no more samples than the number that could be counted in 30 minutes. Typical calculations for X, Y, and Z in a standard and 2 tumor pieces are shown in table 2 to illustrate the kind of values obtained in practice. We obtained the percentage of the injected isotopic dose for each isotope by dividing the calculated value for the isotope in a sample by the calculated value for the 1 percent standard.

Rats used for assay.—The hepatoma-bearing and normal animals were male Sprague-Dawley rats of the Charles River variety which had been fed either a diet containing 0.036 percent *N*-2-fluorenylacetamide for 15 weeks and a normal diet for about 30 additional weeks to obtain liver tumors, or a normal diet for control purposes (*5*). After the rats received intravenous injections of 4.0 ml of the triadic labeled proteins they were kept for 17 to 24 hours and killed under Nembutal anesthesia by total retrograde gravity perfusion with 0.9 percent NaCl (after removal of blood samples for assay). Samples of perfused tissue weighing about 1 g were taken for counting. All parts of all tumors, livers, kidneys, spleens, lungs, hearts, and thyroids were assayed.

Reproducibility.—The same procedures as outlined for the paired label (*1*) were used to determine the reproducibility of the triadic label method.

TABLE 2.—Simultaneous count rates in 3 channels (J, K, and L) of a mixture of 3 radioiodine isotopes in 3 typical samples, and results of simultaneous solution for the total count rate of each isotope preparation (X, Y, and Z) in all 3 channels

		Samples*		
		Number 1	Number 2	Number 3
Channel count rates†	J	7740	660	3690
	K	24500	2270	8320
	L	57200	9390	22800
Count rates of isotope preparations‡	X	22900	1970	11600
	Y	41500	3340	10300
	Z	25000	7010	12900

*Sample 1 was a standard for 1 percent of the injected dose in experiment 2; samples 2 and 3 were tumor pieces #19 and #38 of rat #23-12 in experiment 2.

†Channel J counted integrally above 0.65 Mev; channel K counted differentially between 0.56 and 0.50 Mev; channel L counted differentially between 0.39 and 0.33 Mev. Count rates are corrected for background.

‡X is count rate in all channels due to I^{133} preparation; Y is count rate in all channels of I^{133} preparation; Z is count rate in all channels of I^{131} preparation. *Note* that $J + K + L = X + Y + Z$.

When a mixture of the 3 isotopes was used to label a single antibody globulin with known kidney-localizing activity, all 3 labels were found to be present to the same extent (in terms of the percentage of injected isotopic dose to 2 significant figures) in all tissues of rats receiving the trilabeled protein. High localization in kidney indicated preservation of antibody activity; low and equivalent localization in thyroid (less than 2% of the dose 24 hours after injection in rats not on dietary iodide) revealed biological stability of the iodinated protein; high and equivalent blood values revealed further biological stability of the protein and low degree of denaturation.

When another portion of the antikidney antiserum globulin was divided in three parts and each part was iodinated with a different isotope, the recombined parts showed no greater divergencies in localization properties among themselves *in vivo* than were observed when the same isotope (I^{131}) was coupled several different times (5%).

Limitations.—It has already been pointed out in the section on radioiodination of antiserum globulins that the amount of I^{130} from the Oak Ridge product which could be coupled safely to antiserum globulin was limited because of carrier present, typically to 0.02 mc I^{130}/20 mg globulin as of the day of assay. Because of the spectral overlap of I^{133} (table 1) in channel J (primarily for counting I^{130}) and, further, because of the spectral overlap of I^{131} in channel K (primarily for counting I^{133}), the amounts of I^{133} and I^{131} to be used with I^{130} as a triad were also limited to the same order of magnitude as the I^{130}. The actual amounts of radioactivity to be coupled to the 3 proteins on the day of injection were determined, therefore, by the expected final ratios of the isotopes in the samples to be counted on the day of assay and the age of the I^{133}. This required a number of preliminary trials to bring the ratios within the required limits. Errors in measurement varied as the isotope ratios varied and had to be determined for each sample. Such errors are not

included in the tabulations of data in this paper because of the additional complexity they would add to data already necessarily complex.

RESULTS

Comparison Between Antibodies Prepared Against Hepatoma Sediment and Those Against Sediment of Normal-Appearing Portions of Hepatoma-Containing Rat Liver

We wanted to know whether antibodies, in a pool of antiserums prepared against the hepatoma portions of hepatoma-containing rat liver, would localize in hepatomas to a greater extent than would antibodies in a pool of antiserums prepared against the normal-appearing liver portions. We also wanted to know whether the antiliver antibodies would localize in liver to a greater extent than the antihepatoma antibodies. The antitumor antiserum globulin labeled with I^{131}, the antiliver antiserum globulin with I^{130}, and normal rabbit serum globulin with I^{133} were mixed and injected in 3 rats bearing well-defined hepatomas. The results of the interorgan assay are presented in 4 forms (table 3): the percentage of the injected isotopic dose found in whole tissue, the percentage per g of tissue, the net percentage of the injected antibody globulin dose (corrected for background localization of normal globulin) in whole tissue, and the net percentage per g. The net values for the tissues are more uniform than the total values uncorrected for background. No appreciable difference between the localizing properties of the 2 antiserum globulins can be seen.

The ratio of the net percentage dose of the antitumor globulin to that of the net percentage dose of the antiliver globulin was also calculated for each 1 g piece of tumor and liver. The distributions of the ratios for all the pieces in the 3 tumors and 3 livers are shown in table 4 under the heading "Antihepatoma/antiliver." Again no appreciable difference in the distributions of the two antiserum globulins can be seen.

Comparison Between Antibodies Prepared Against Rat Hepatoma Sediment and Those Prepared Against Rat Plasma and Rat Serum

In a previous report (*5*) we had shown that antifibrinogen antibodies rarely localized in very slowly growing (10 g) liver tumors induced by fluorenylacetamide. This contrasted greatly with the extensive localization of antifibrinogen antibodies in rapidly growing transplanted tumors of similar size which we had previously observed (*3*, *4*) and which Spar, Bale, and Goodland (*8*) similarly had found. Spar *et al.* (*9*) also had found later that antifibrin antibodies did not localize as a rule in spontaneous dog tumors, though an exception occasionally occurred. Since we found that the nonspecific background localization varied extensively in many induced rat hepatomas within the liver, we were unable to

TABLE 3.—Localization of I^{131}-antihepatoma antiserum globulin-pool-I, I^{130}-antiliver antiserum globulin-pool, and I^{133}-normal globulin 1 day after simultaneous intravenous injection in rats bearing hepatomas induced by *N*-2-fluorenylacetamide

Rat number	Weights (g)			Type of value	Isotope member of triad	Percentage injected isotopic dose*					
						In whole perfused tissues				Per g tissue	
	Whole rat	Hepatoma	Liver			Hepatoma	Liver	Kidney	Spleen	Tumor	Liver
25–9	535	17.5	24.3	Total	131	2.9	2.9	0.70	0.28	0.17	0.12
					130	2.7	2.7	0.50	0.27	0.15	0.11
					133	1.7	1.1	0.23	0.13	0.099	0.047
				Net	131	1.2	1.8	0.47	0.15	0.066	0.073
					130	0.92	1.6	0.27	0.14	0.052	0.066
20–13	575	13.0	27.9	Total	131	2.1	3.1	1.10	0.26	0.16	0.11
					130	2.0	3.0	0.81	0.23	0.16	0.11
					133	0.88	1.5	0.54	0.11	0.067	0.053
				Net	131	1.2	1.6	0.56	0.15	0.094	0.058
					130	1.2	1.6	0.27	0.12	0.090	0.055
21–1	571	13.6	20.4	Total	131	3.4	4.4	0.65	0.40	0.25	0.22
					130	3.1	4.5	0.35	0.42	0.23	0.22
					133	2.1	2.6	0.14	0.17	0.16	0.13
				Net	131	1.2	1.8	0.51	0.23	0.090	0.089
					130	1.0	1.9	0.21	0.25	0.075	0.093

*In whole thyroid the amount localized in each animal for each isotope averaged 1.6 ± 0.3 percent of the injected dose. The amount localized per ml of blood averaged 1.13 ± 0.07 percent.

TABLE 4.—Distribution of ratios of localized antihepatoma antibodies to localized antiliver antibodies* and to localized antifibrin antibodies† in 1 g portions of hepatomas and livers

Range of ratios of antibody pair	Number of 1 g pieces of tissue containing the pairs to antibodies within range of ratio			
	Antihepatoma/antiliver*		Antihepatoma/antifibrin†	
	Hepatoma	Liver	Hepatoma	Liver
0. 0–0. 4	0	0	12	0
0. 9–1. 0	5	11	0	0
1–2	35	60	6	0
2–3	2	2	6	0
3–4	0	0	6	0
4–5	0	0	8	1
5–10			24	27
10–15			4	41
15–20			2	6
20–25			4	8
25–30			0	3
30–300			8	0
300–			8	0
Total	42	73	88	86

*In the hepatomas and livers of the 3 rats described in table 3. Ratios were calculated from:

$$\frac{\text{Percent } I^{131}\text{-antihepatoma minus percent } I^{131}\text{-normal}}{\text{Percent } I^{130}\text{-antiliver minus percent } I^{131}\text{-normal}}.$$

†In the hepatomas and livers of the first 4 rats of table 5. Ratios were calculated from:

$$\frac{\text{Percent } I^{131}\text{-antihepatoma minus percent } I^{131}\text{-antirat serum}}{\text{Percent } I^{130}\text{-antirat plasma minus percent } I^{131}\text{-antirat serum}}.$$

measure simultaneously the localizing properties of antitumor and antifibrin antibodies in such tumors until the triadic label technique had been developed.

In experiment 2 we compared the localizing properties of antibodies from antitumor antiserum-pool-2 and antibodies from the antirat-plasma antiserum known to contain an appreciable amount of antifibrinogen antibody (fibrin-reacting antibodies). For control the globulin fraction of an antirat-serum antiserum was used. The data in table 5 show the localization properties of the triad in 4 tumor-bearing and in 3 normal rats. The former were chosen to give data on a wide range of tumor sizes from 3 to 45 g. On a whole-organ basis, antiplasma localized slightly more in all tumors and livers than the control globulin. The antihepatoma antibody localized to a much greater extent in the hepatomas of the tumor-bearing rats and in the liver, kidneys, and spleens of both tumor-bearing and normal rats. The data in table 4 (antihepatoma/antifibrin) show, however, that the ratio between localizing antitumor antibody and localizing antiplasma antibody (corrected for background localization) varies very widely from one portion of tumor to the next, whereas in the liver portions (whether from tumor bearers or normal rats) the ratio has a narrower range. From the wide variation in ratio

in the hepatoma it is evident that the 2 antibody preparations are directed by and large against different components.

TABLE 5.—Localization of I^{131}-antihepatoma antiserum globulin-pool-2, I^{130}-antirat-plasma antiserum globulin, and I^{133}-antirat-serum antiserum globulin 1 day after simultaneous intravenous injection in rats bearing tumors induced by *N*-2-fluorenyl-acetamide and in normal rats

Rat number	Weight (g)			Isotope member of triad	Percentage of the injected isotopic dose in whole perfused tissues*			
	Whole rat	Hepatoma	Liver		Hepatoma	Liver	Kidney	Spleen
25-17	549	3. 17	27. 0	131	0. 88	6. 3	1. 3	0. 51
				130	0. 51	1. 4	0. 08	0. 13
				133	0. 34	0. 96	0. 08	0. 08
25-4†	540	11. 4	21. 5	131	2. 3	5. 6	0. 98	0. 44
				130	1. 4	1. 3	0. 09	0. 10
				133	1. 2	0. 95	0. 08	0. 07
23-15	571	28. 6	24. 8	131	5. 4	4. 6	1. 01	0. 32
				130	3. 1	1. 1	0. 12	0. 10
				133	2. 3	1. 0	0. 10	0. 06
23-12‡	485	45. 2	12. 4	131	10. 6	2. 9	0. 98	0. 44
				130	6. 7	0. 90	0. 09	0. 10
				133	5. 0	0. 67	0. 08	0. 07
C5-3	513	—	17. 8	131	—	5. 9	1. 4	0. 59
				130	—	1. 2	0. 11	0. 16
				133	—	0. 81	0. 09	0. 10
C5-4	455	—	20. 2	131	—	5. 7	1. 3	0. 64
				130	—	1. 5	0. 10	0. 18
				133	—	0. 72	0. 12	0. 12
C5-5	520	—	20. 0	131	—	6. 7	1. 2	0. 56
				130	—	1. 7	0. 09	0. 17
				133	—	1. 1	0. 09	0. 10

*Average localization of all isotopes in thyroids of all animals was 1.6 ± 0.4 percent of the injected isotopic dose. The average localization per ml of blood was 1.01 ± 0.08 percent.

†*See* table 6 for further data for this rat.

‡*See* text-figure 1 for further data for this rat.

Sometimes rats are found with tumors not only in the liver but also in one or more other sites. Such a rat was #25-4 which had a 14 g primary carcinoma of the intestine in addition to a well-defined 11 g liver tumor (histologically, hepatoma, type II). The data in table 6 show the localization properties of antitumor and antiplasma preparations in the 2 tumors. The net values, corrected for background (antirat serum antibody), reveal that the observed high localization of antihepatoma antiserum globulin in the intestinal carcinoma was not due to antibody localization but to a high nonspecific background. Moreover, the intestinal tumor did not localize any antifibrinogen antibody from the antirat-plasma serum. If the antihepatoma and antiplasma preparations had been assayed as a pair without further control, we might have been led to believe that the 2 tumors did indeed localize the antitumor antibodies equally well and that the carcinoma of the intestine localized antiplasma

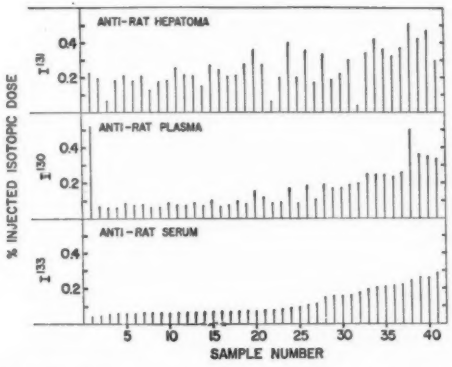
antibodies to an even higher extent than did the hepatoma. This example emphasizes the need of the triadic label for controlled determinations of antibodies in unexplored situations.

TABLE 6.—Differential localizations of antitumor (I^{131}) and antifibrin (I^{130}) antibodies above background localization (I^{133}) in different tumors within the same individual*

Tissue	Weight (g)	Percentage injected isotopic dose per g				
		Total dose per g			Net dose per g†	
		131	130	133	131	130
Carcinoma of intestine	13. 6	0. 21	0. 22	0. 20	0. 008	0. 014
Hepatoma	11. 4	0. 21	0. 12	0. 10	0. 10	0. 021
Liver	21. 5	0. 26	0. 059	0. 044	0. 21	0. 015
Kidneys	4. 4	0. 22	0. 019	0. 018	0. 20	0. 001
Spleen	1. 1	0. 42	0. 098	0. 062	0. 35	0. 036

*See table 5 for other data for this rat.
†Total percent dose per g minus percent dose per g of I^{133} globulin.

When a tumor becomes large, a variety of histological situations exist: Some portions perfuse better than others; some are quite necrotic, even fluid, while others are firm and solid; some contain more stroma than others; and some are borderline neoplastic areas of bile-duct proliferation or cirrhosis. It was important to see if the triadic label would permit assay of antitumor antibodies even in as extreme a case as the 45 g tumor in rat #23-12. The distribution of the 3 labels in individual 1 g portions of the 45 g tumor are shown graphically in text-figure 1 where the data for the pieces are arranged in order of increasing background



TEXT-FIGURE 1.—Distributions of antitumor (I^{131}) and antifibrin (I^{130}) antibodies in 1 g pieces of an extremely large hepatoma as compared with the distribution of nonspecific globulin (I^{133}). Pieces are arranged in order of increasing nonspecific background localization. *See* table 5 for further data (rat #23-12).

localization. Examples of contrasting levels of localization can be seen. Whereas the antitumor antiserum globulin localized to a greater extent than the other proteins in nearly all instances, there were situations in

which the reverse occurred. Extreme cases were sample 1, which contained a fresh, well-formed blood clot, sample 32 which was mainly fluid, and sample 41 which resisted perfusion.

DISCUSSION

Use of autochthonous tumors in laboratory animals for an experimental study presents more difficult problems than the use of the transplanted variety, but the problems, at least, are the same as must be met in human cancer studies. Like human tumors, autochthonous animal tumors of a given histological type, spontaneous and induced, are much more variable in size, shape, site, growth rate, etc. from one laboratory animal to the next than are the more uniform transplanted growths. In each new or unusual case, background localization must be determined at the time of antibody assay. By the use of multiple labels, as in paired label studies, we can solve the problems of background localization by making the individual serve as his own control subject. Moreover, small subdivided portions of tumor tissue can be assayed for background and antibody localizations to take care of any complicating features such as necrosis, thrombi, varying degrees of vascularity, and lesions in general. The triadic label has the further advantage over the paired label in allowing the comparison of two antiserums on the basis of net localization over and above background. In human cancer this can be done, as it is now feasible with autochthonous animal cancer, only by the assay of all preparations simultaneously. The use of multiple labels makes this possible.

If one were to use only a single label in the general study of autochthonous tumors and found an occasional tumor with a high localization of labeled antiserum globulin, one would not know whether this high value were due to localization of antibody, *per se*, or due merely to a high background localization. Thus, in one of our experiments (table 6) it would appear that we had obtained localization of antifibrin antibodies in the intestinal carcinoma on the basis of previous experience with hepatomas. Actually we were able to attribute this particular high localization completely to high background. The failure to control background by a paired label technique made it impossible for Spar *et al.* (*9*) to reach any definite conclusions about the localization of antifibrin antibodies in the spontaneous dog tumors which they studied.

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Synergism Between Small Doses of Total-Body and Local X Radiation to Transplanted Mouse Tumors [1, 2]

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SUMMARY

A synergistic effect between total-body and local X-ray exposures was demonstrated in 7 transplanted tumors. The magnitude of the enhanced regression obtained with systemic irradiation varied with different tumors. The relations between the degree of synergism and the characteristics of the tumors have been considered. In general, the susceptibility to enhancement was greatest in the more radiosensitive and curable tumors. The possibility of the relations of this phenomenon to alterations in the immune process has also been considered. It was concluded that a satisfactory explanation on this basis was unlikely.—J. Nat. Cancer Inst. 26: 1335-1347, 1961.

THE USEFULNESS of whole-body irradiation in the clinical treatment of leukemia has been recognized for over 30 years. Recently, Hollcroft *et al.* (*1–3*) reported on the effects of a small increment of total-body irradiation on the response of experimental tumors, other than leukemias, treated with local X radiation. They demonstrated that the oncolytic effects of tumor irradiation could be increased by the concomitant administration of whole-body irradiation in the 2 tumors that they used—the L-1 lymphosarcoma and the C3HBA adenocarcinoma. They further found that 50 r of total-body exposure produced a maximum effect on the tumor; larger doses of radiation did not bring about any greater regression.

This relationship has obvious theoretical importance in the production of such a pronounced effect by relatively low doses of radiation. There is also a potential clinical applicability of this phenomenon. Since, to our knowledge, these results had never been confirmed, we repeated this work and have confirmed the reports of Hollcroft and her collaborators. We also studied several other transplanted tumors with a spectrum of other characteristics to determine whether this synergism was a general

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or a specific phenomenon and under what conditions it was found. A preliminary report of our findings in 3 of these tumors was presented as an introduction to other studies from this laboratory (*4*). This paper is a detailed report of our results and their pattern in the whole array of tumors studied.

MATERIALS AND METHODS

Tumors in the active growth phase were removed from carrier mice and cut into small fragments under aseptic conditions. These cuttings were implanted subcutaneously with a modified #13 B-D hypodermic needle into the dorsolateral aspect of the lower thigh of recipient mice. After the tumors had grown to slightly less than 1 cm in diameter, the experimental mice were divided into 16 groups of 10 or more animals. The 4 most sensitive tumors were treated with total tumor doses of 800, 1400, and 2000 r distributed as shown in table 1; with the 3 most resistant tumors, the 800 r dose was not used and the 2800 r level added. The actual radiation doses and the strain and source of the mice used for each tumor are indicated in table 3. Within each classification the same total dose was administered to the tumor, the local dose being decreased to compensate for the added increment of total-body exposure.

TABLE 1.—Experimental design

Whole-body dose (r)	Local radiation to tumor (r)				
0	0	800	1400	2000	2800
50	0	750	1350	1950	2750
100	0	700	1300	1900	2700
200	0	600	1200	1800	2600
Total radiation to tumor (r)	0, 50, 100, 200	800	1400	2000	2800

The exposures were alternated between 2 superficial X-ray units (Keleket and Picker), each operated at 120 kvp (HVL = 2.0 mm Al) and with similar outputs, approximating 700 r per minute. The radiation was always administered with the mice enclosed in a ⅛-inch lead box from which only the hind leg protruded. The extraneous exposure was further limited by collimation of the X-ray beam. Total-body irradiation was administered within 1 minute of completion of the local exposure by use of the same machines with the cones and external shielding removed and with a greater target-skin distance.

The contribution of scattered radiation during the local exposure to the whole-body dose was determined with a Victoreen r-meter, a frozen mouse used as a phantom. The scattered radiation at the level of the bladder was 0.9 percent of the local exposure, while at the head it was less than 0.1 percent. The scatter at the xiphoid process, which gives an approximation of the average scattered dose received by the whole body,

was 0.4 percent. The average whole-body component of a local exposure ranged from 3.2 r with 800 r to 11.2 r, with a 2800 r local dose.

These techniques represent a departure from those used by Hollcroft *et al.* In their experiments, the tumor was implanted in the side rather than in the hind leg. Also, the local systemic exposures were administered simultaneously, while the differences in local and whole-body doses were accomplished through suitable shielding. Their results (*2*) indicated, however, that the simultaneous administration of the radiation exposures was not necessary, though the synergism was gradually lost with the passage of time. As will be shown, the results obtained in this study were essentially the same as those of Hollcroft and associates in the two tumors used in common. This indicates that this phenomenon is not restricted to certain sites of implantation nor is it dependent on the simultaneous administration of the two exposures.

Calculations.—The tumors were measured in 3 dimensions with calipers before the initiation of treatment and at frequent intervals thereafter. The product of these 3 measurements was taken as an approximation of tumor volume. The tumor size at any subsequent time was expressed as the percent of the volume at the time of irradiation (PS_o).

$$PS_o = \frac{\text{volume at time } t}{\text{volume at irradiation}} \times 100$$

This value was determined for each measurement and group averages calculated at serial times after irradiation. Each average PS_o was compared to the corresponding average for the untreated control group to obtain the percentage inhibition of growth (PI).

$$PI = \frac{PS_o \text{ (control)} - PS_o \text{ (experimental)}}{PS_o \text{ (control)}} \times 100$$

The extent of inhibition obtained with each increment of total-body irradiation was compared to the inhibition obtained with only local tumor exposure in terms of the percent enhancement of effect (PE). This was calculated from the maxima of the smoothed curves of percentage inhibition plotted as a function of the time after irradiation.

Complete, though perhaps temporary, regression of all tumors of a group would give a PI value of 100 percent at the maximum of the curve. The effect of whole-body (*W.B.*) exposure can be evaluated as the fraction of this undone-oncolysis accomplished by the use of this exposure. It would be calculated as:

$$PE = \frac{[100 - PI \text{ (No } W.B.)] - [100 - PI \text{ } (W.B.)]}{[100 - PI \text{ (No } W.B.)]} \times 100$$

Maximum inhibition of growth occurred at about the same time for all whole-body doses used at any given total tumor dose. For each tumor these times, or the best approximation of single times for each tumor dose, were selected by inspection of the curves. With the individual

measurements of PS_o at this time, the actual mean tumor sizes of the groups that received whole-body irradiation were tested against the corresponding group that received only local irradiation with the usual t test. The values of PE indicated as statistically significant in table 3 thus refer to differences between group means at the time of maximum inhibition. In text-figures 1 to 7 these groups are indicated by heavy lines.

RESULTS

The tumor volumes, expressed as PS_o, are presented in table 2 at representative times after irradiation. The results obtained when PI, as calculated from these and additional data, was plotted as a function of time after irradiation are shown in the text-figures. The values of PE derived from these smoothed curves are summarized in table 3.

The reader may use the data of table 2 to confirm the measures of inhibition shown in the text-figures and the measures of enhancement shown in table 3. The measure of inhibition on a day due to treatment is the percentage by which the treated tumor volume, adjusted for its initial size, is less than the untreated volume. Where table 3 shows significant enhancement[4] due to whole-body irradiation, it can be recognized in table 2 that tumor volumes with combined radiation treatment are smaller than when only local radiation is administered.

TABLE 2.—Tumor volumes at representative times after irradiation, expressed as PS_o

Tumor	Total tumor dose (r)	Whole-body dose (r)	Volume (cm^3) at irradiation	Days after irradiation					
				3	7	10½	14½	17½	21½
L-1 lymphosarcoma	0	0	2.1	261	615	848	1079		
	50	50	2.3	239	584	762	851		
	100	100	2.6	219	541	714	—		
	200	200	1.8	153	477	781	1002		
	800	0	2.0	53	170	394	528		
		50	1.8	55	242	361	538		
		100	2.4	67	215	523	530		
		200	2.0	49	136	400	621		
	1400	0	2.0	37	76	227	297		
		50	2.1	35	10	11	24		
		100	1.7	35	30	74	174		
		200	2.1	37	6	7	11		
	2000	0	2.0	41	59	108	222		
		50	2.3	30	3	2	12		
		100	1.8	25	4	91	218		
		200	2.4	29	14	28	87		

[4] Enhancement, on a day, due to whole-body irradiation may be thought of as the percentage by which the tumor volume (adjusted for its initial size), obtained when both local and whole-body irradiation are administered, is less than the corresponding volume when only local radiation in the same total dose is administered. The percentage enhancements shown in table 3 do not correspond strictly to those calculated on a particular day. Instead, those in table 3 are based on the maximum percentage inhibitions due to treatment; they will, however, correspond approximately to the daily enhancement values which can be gleaned from table 2.

TABLE 2.—Tumor volumes at representative times after irradiation, expressed as PS_0 —Continued

Tumor	Total tumor dose (r)	Whole-body dose (r)	Volume (cm^3) at irradiation	Days after irradiation					
				3	7	10½	14½	17½	21½
C3HBA adenocarcinoma	0	0	3.5	139	200	325	484	551	597
	50	50	2.4	140	220	286	409	449	431
	100	100	3.1	142	201	274	348	387	688
	200	200	2.2	141	214	266	369	424	762
	1400	0	3.4	107	140	213	286	382	371
		50	2.8	113	128	147	117	200	242
		100	3.2	119	149	173	216	216	383
		200	2.9	119	129	154	209	255	360
	2000	0	2.8	127	149	185	252	222	316
		50	2.5	112	119	151	199	207	301
		100	2.5	120	139	164	196	225	318
		200	2.2	113	115	127	149	181	252
	2800	0	2.5	103	98	79	100	114	154
		50	2.3	110	115	127	151	170	204
		100	2.5	101	103	118	122	131	156
		200	2.2	105	102	113	125	137	287
Ehrlich carcinoma	0	0	2.1	156	237	336	464	516	597
	50	50	2.0	162	267	396	545	706	767
	100	100	2.6	160	264	358	566	549	743
	200	200	2.4	125	189	254	313	402	509
	800	0	1.6	139	210	308	410	487	499
		50	2.0	141	183	209	385	491	536
		100	2.5	136	171	212	298	448	579
		200	2.2	141	192	231	321	429	455
	1400	0	2.5	142	155	203	259	301	473
		50	2.2	110	110	108	147	212	230
		100	2.5	117	105	111	148	216	206
		200	2.4	128	144	152	176	235	364
	2000	0	1.9	175	117	304	148	151	206
		50	1.8	150	156	155	163	198	303
		100	2.1	132	111	101	100	177	212
		200	2.0	122	151	111	118	154	183
Krebs-2 carcinoma	0	0	2.6	139	213	313	441	468	585
	50	50	2.8	142	216	323	501	598	763
	100	100	3.3	142	190	251	365	421	508
	200	200	2.2	127	189	242	338	438	314
	800	0	2.5	127	134	194	284	380	576
		50	3.5	117	146	210	297	361	423
		100	2.8	123	149	180	237	276	343
		200	2.7	133	170	220	316	361	410
	1400	0	2.3	116	137	164	213	269	344
		50	3.4	107	116	136	149	196	247
		100	4.2	132	129	163	178	194	263
		200	2.9	102	111	125	157	198	225
	2000	0	2.6	112	116	141	187	205	231
		50	3.0	108	115	137	159	273	247
		100	2.2	107	117	125	163	171	189
		200	2.7	108	93	183	130	139	158

TABLE 2.—Tumor volumes at representative times after irradiation, expressed as PS_0 —Continued

Tumor	Total tumor dose (r)	Whole-body dose (r)	Volume (cm^3) at irradiation	Days after irradiation					
				3	7	10½	14½	17½	21½
Sarcoma 37	0	0	2.3	158	241	326	348	466	379
	50	50	2.6	149	222	281	409	—	—
	100	100	2.3	157	238	276	352	—	—
	200	200	2.4	154	190	218	264	—	—
	800	0	2.5	133	158	226	300	356	369
		50	2.3	150	161	238	321	362	248
		100	2.3	145	167	225	232	337	287
		200	2.5	119	124	133	209	198	320
	1400	0	2.6	114	129	166	235	236	194
		50	2.2	109	130	130	174	176	206
		100	2.6	106	117	128	159	150	151
		200	2.1	116	121	120	108	212	262
	2000	0	2.3	108	93	107	136	176	126
		50	2.1	105	103	88	97	89	81
		100	2.3	117	99	109	115	61	49
		200	2.5	104	74	71	66	48	64
H-P melanoma	0	0	1.8	124	196	276	349	504	744
	50	50	1.4	138	222	412	696	843	880
	100	100	1.8	122	237	354	517	546	568
	200	200	1.6	111	185	267	273	411	601
	1400	0	1.8	88	120	146	230	272	343
		50	1.5	112	152	167	207	237	232
		100	1.7	105	133	157	206	330	388
		200	1.4	96	129	155	182	209	256
	2000	0	1.6	88	104	114	127	181	212
		50	1.8	79	96	106	122	144	214
		100	1.5	87	97	91	95	121	130
		200	1.5	90	122	137	206	247	406
	2800	0	2.3	86	108	66	85	80	117
		50	2.0	97	109	112	106	157	191
		100	1.4	104	130	96	118	123	104
		200	1.9	98	135	166	66	125	59
C-1300 neuroblastoma	0	0	1.3	168	290	335	399	555	563
	50	50	1.5	153	229	279	281	399	434
	100	100	1.7	145	204	282	326	333	494
	200	200	1.5	162	232	279	394	535	740
	1400	0	1.4	115	125	173	223	252	272
		50	1.9	129	128	176	256	292	322
		100	1.5	111	110	143	186	259	352
		200	1.5	108	110	154	191	202	188
	2000	0	1.7	118	107	142	191	249	294
		50	1.5	109	97	120	127	173	165
		100	1.5	101	74	93	126	138	126
		200	1.3	97	95	93	113	156	172
	2800	0	1.2	104	62	52	63	76	103
		50	1.5	95	66	46	60	58	75
		100	1.6	100	68	52	46	85	74
		200	1.8	103	59	56	106	135	180

TABLE 3.—*PE* obtained by various increments of whole-body irradiation in the 7 tumors studied

Tumor	Whole-body dose (r)	Total tumor dose (r)			
		800	1400	2000	2800
L-1 lymphosarcoma (Hollcroft—NCI)† A/Jax‡	50	0	95*	100*	
	100	0	79*	90*	
	200	14	100*	80*	
C3HBA adenocarcinoma (Barrett—NCI) C3HeB/Jax	50		42*	21	0
	100		37	23	0
	200		30	35*	0
Ehrlich carcinoma (Prychodko—Detroit Inst. Cancer Res.) Swiss Webster/Rockland	50	21*	59*	0	
	100	17*	54*	15	
	200	12*	43*	4	
Krebs-2 carcinoma (Patt-Argonne National Lab.) Swiss Webster/Rockland	50	0	27*	35	
	100	13*	7	45	
	200	0	26*	56*	
Sarcoma 37 (R. B. Jackson Memorial Lab.) Swiss Webster/Rawley	50	0	9	30*	
	100	0	21*	39*	
	200	36*	36*	55*	
Harding-Passey melanoma (R. B. Jackson Memorial Lab.) Swiss Webster/Rawley	50		10	9	18
	100		0	31*	39
	200		16	0	61*
C-1300 neuroblastoma (R. B. Jackson Memorial Lab.) A/Jax	50		0	11	12
	100		12	30	6
	200		12	24	0

*Indicates statistical significance (*see* text).

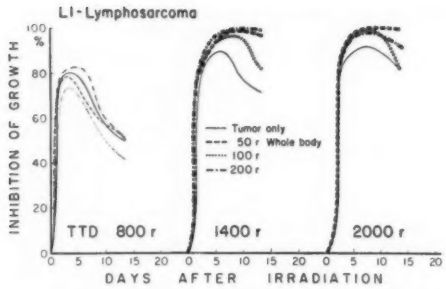
†Source of tumor. (The authors appreciate the kindness of the individuals and organizations that supplied these transplanted tumors.)

‡Strain of mouse and source: Jax = Jackson Memorial Laboratory, Bar Harbor, Me. Rockland = Rockland Farms, New City, N.Y. Rawley = Roy Rawley Farms, Plymouth, Mich.

The data in table 2 indicate that whole-body irradiation alone had little effect on tumor growth. In some instances, however, a systemic exposure to 200 r did produce a small inhibition of growth. Even a 200 r dose apparently had no deleterious effects on the experimental mice beyond those ordinarily associated with the growing tumor.

When the L-1 lymphosarcoma, 1 of the 2 tumors previously studied by Hollcroft *et al.*, was used (text-fig. 1) a total tumor dose of 800 r produced a substantial inhibition of growth. At this level, the use of an increment of whole-body irradiation did not affect the amount of inhibition obtained, as the 4 curves are almost superimposed. This resulted in *PE* values approximating zero. At the 1400 r level, local exposure of the tumor produced about 90 percent inhibition of growth. Even at this high level of inhibition, the partial replacement of local exposure by total-body irradiation brought about a statistically significant increase in *PI* and, thus, enhancement. This was again true at the 2000 r level where the values of *PE* were substantial and statistically significant.

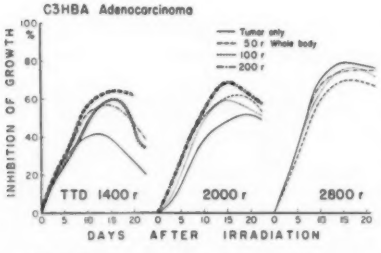
Somewhat comparable results were obtained with the C3HBA adenocarcinoma, the other tumor investigated by Hollcroft *et al.* The curves



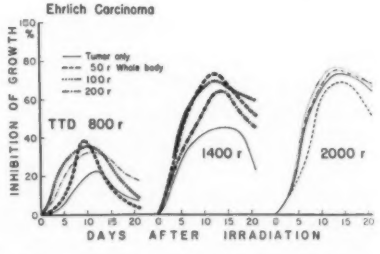
TEXT-FIGURE 1.—Dose-response curves of the L-1 lymphosarcoma.

obtained with any increment of total-body irradiation (text-fig. 2) lay substantially above that resulting from 1400 r directly to the tumor. These 3 curves were essentially the same. The relatively low absolute amount of inhibition obtained with local irradiation gave rise to moderately high values for *PE*. The picture was similar at the 2000 r total-tumor dose. A substantially higher level of inhibition was obtained with administration of 2800 r to the tumor. At this increased dose level there was no further inhibition of growth obtained with part of the local exposure replaced by systemic radiation.

As shown in text-figure 3 and table 3, the results obtained with the Ehrlich carcinoma were much the same in that it was not possible to achieve further enhancement of effect by systemic exposure at the highest total tumor dose examined. However this failure of potentiation took place at the 2000 r level, whereas, with the previous tumor, potentiation was present at this dose and was not lost until 2800 r was used.



TEXT-FIGURE 2.—Dose-response curves of the C3HBA adenocarcinoma.

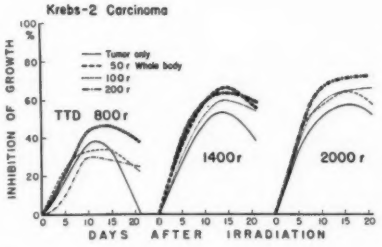


TEXT-FIGURE 3.—Dose-response curves of the Ehrlich carcinoma.

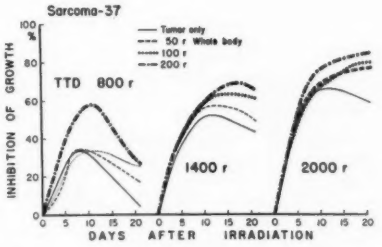
With the Krebs-2 carcinoma (text-fig. 4) there was no greater inhibition of growth with an increment of 50 or 200 r of whole-body exposure than when 800 r was administered directly to the tumor. A statistically significant *PE* was obtained with 100 r of total-body radiation at the same total dose level, though the numerical value was only 13. However, at the 1400 r level, this dose produced substantially less potentiation than did

the other two doses of total-body radiation. At a total tumor dose of 2000 r, only 200 r of systemic exposure had a significant effect.

A single one of the whole-body curves again showed an aberrant rise at the 800 r level with the Sarcoma 37 (text-fig. 5); in this instance the curve was for 200 r. Because of the relatively low level of inhibition obtained with this tumor dose, moderately large value of *PE* resulted. At this tumor-dose level, the other 2 whole-body curves did not differ from that obtained with tumor irradiation alone. At the 1400 and 2000 r tumor-dose levels, a good separation between the curves for whole-body and for tumor exposure alone were obtained, with clear-cut values of *PE*.



TEXT-FIGURE 4.—Dose-response curves of the Krebs-2 carcinoma.

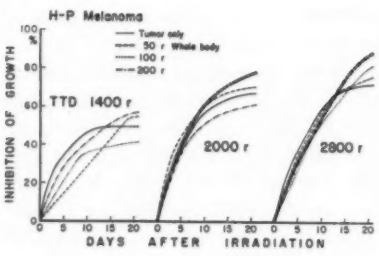


TEXT-FIGURE 5.—Dose-response curves of the Sarcoma 37.

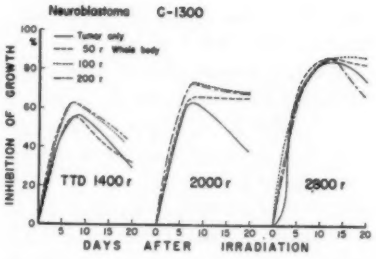
Study of the Harding-Passey melanoma and the C-1300 neuroblastoma revealed a different pattern of effect than was seen with the previously considered tumors. With the first-mentioned tumor (text-fig. 6) fairly high levels of *PI* were obtained, though very little difference was seen between the curves obtained with or without systemic exposure. A statistically significant increase over the results obtained with tumor irradiation alone was found in only two isolated instances: 100 r of total-body radiation with a tumor dose of 2000 r and 200 r whole-body at a tumor dose of 2800 r.

Unexpectedly high levels of growth inhibition were seen in the C-1300 neuroblastoma, as shown in text-figure 7. The maximum level of growth inhibition obtained was dependent only on the tumor dose and was not related to whether or not an increment of total-body irradiation was administered. At the 2000 r level, some difference was seen in that the inhibition was rapidly lost when only direct tumor irradiation was used while the inhibition persisted when a total-body exposure was administered.

The radiation doses used in these experiments were not selected to produce cure of the tumor. In three instances, as shown in table 4, an appreciable number of the tumors regressed completely. Only isolated instances of such regression were seen in the other 4 tumors studied. With the L-1 lymphosarcoma, whole-body irradiation did not effect the cure rate at any tumor dose other than 1400 r. At this level, all 3 doses of systemic exposure produced an appreciable and similar increase in the



TEXT-FIGURE 6.—Dose-response curves of the Harding-Passey melanoma.



TEXT-FIGURE 7.—Dose-response curves of the C-1300 neuroblastoma.

number of tumors cured. The 1400 r dose showed a similar effect in the Ehrlich carcinoma. In addition, in this tumor 100 or 200 r increased the frequency of complete regression at the 2000 r level. There was only a somewhat vague tendency toward increased cure rate with the administration of systemic irradiation to the Sarcoma 37.

TABLE 4.—Percent of mice with complete regression of tumors

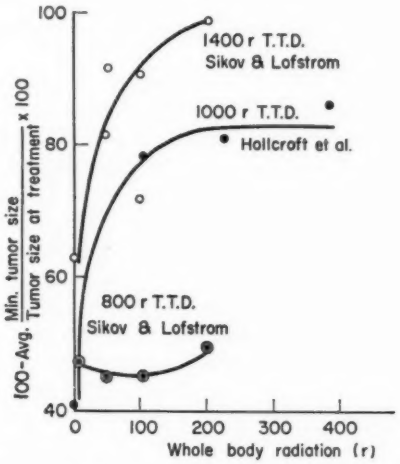
Whole-body dose (r)	Total tumor dose (r)											
	L-1 lymphosarcoma				Ehrlich carcinoma				Sarcoma 37			
	0	800	1400	2000	0	800	1400	2000	0	800	1400	2000
0	0	20	40	80	0	0	0	10	0	15	14	29
50	0	30	80	100	0	10	30	10	7	27	7	46
100	0	20	90	100	0	0	22	38	8	7	22	25
200	0	10	90	90	0	0	25	30	13	23	27	58

DISCUSSION

The results obtained in the present experiments with the L-1 lymphosarcoma and the C3HBA adenocarcinoma may be compared with those obtained by Hollcroft and Matthews (*3*) using the same 2 tumors. The results presented in this paper qualitatively resemble those obtained in the previous studies in that the use of whole-body irradiation increased the oncolysis obtained with local-tumor irradiation and that a whole-body dose of 50 r was sufficient to elicit a maximum effect on the tumor.

The results obtained with the L-1 lymphosarcoma in our experiments have been recalculated on the basis used by Hollcroft. These data for both studies are presented in text-figure 8 where (100–R) is plotted as a function of whole-body dose for various total tumor doses. R is the ratio between the tumor size at its minimum to the size at the time of irradiation. The curve following 1000 r in the work of Hollcroft closely resembles our curve obtained at 1400 r. This line (Hollcroft's) lies intermediate between our curves obtained at 800 and 1400 r, which indicates that the quantitative relations are also the same. There was no decrease

in the size of the C3HBA adenocarcinoma in our experiments so that (100–R) was a negative number and could not be directly compared with the data of Hollcroft *et al.* The qualitative findings were quite similar, as we have indicated.



TEXT-FIGURE 8.—Comparison of data from our studies with those of Hollcroft and Matthews (*5*).

As described under "Materials and Methods," we made two major departures from the techniques used by Hollcroft *et al.* These consisted of implantation of the tumor in the leg rather than in the side and the use of two nonsimultaneous exposures. Despite these differences in technique, the results with the 2 tumors used in common were very comparable. Additionally, we saw similar results with the other tumors used in this study, which indicate that this enhancement of effect through the use of total-body irradiation is a general phenomenon. There are, however, definite limitations on the situations in which this generalization is applicable in terms of the tumors as well as the dosage range.

Throughout our experiments we saw that there were certain tumors, such as the L-1 lymphosarcoma, in which systemic irradiation failed to affect potentiation until some certain level of direct tumor irradiation had been attained. Conversely, with other tumors such as the Ehrlich carcinoma, there was no further potentiation above moderate (or high, in some cases) levels of local exposure. We have not been able to derive any pattern as to where these lower or upper limits to effectiveness pertain and are therefore unable to offer any explanation of this relation.

Similarly, we observed that the degree of effectiveness of total-body exposure, whether measured in terms of absolute increases in *PI* or *PE*, varied from tumor to tumor. Since the effect depended so markedly on the tumor dose, it was difficult to rank in a purely quantitative fashion the susceptibility to enhancement by whole-body irradiation. When the

tumors were arrayed in a somewhat arbitrary manner, however, certain patterns were discernible. As was shown previously, the Harding-Passey melanoma and the C-1300 neuroblastoma were the least responsive to enhancement of effect, whether measured in terms of *PE* or as absolute increases in *PI*. These 2 tumors also were the least responsive to irradiation, as evidenced by the relatively low values of *PI* obtained at any tumor dose.

Further, these histological types are usually considered to have low clinical irradiation curability. The highly sensitive L-1 lymphosarcoma was quite susceptible to the potentiating effects of the systemic exposures. This tumor showed the greatest *PE* values obtained, even though the amount of increase in *PI* possible was limited by the fact that whole-body irradiation brought about complete cure in a large fraction of the mice. Similarly, the influence of total-body irradiation on the Sarcoma 37 and the Ehrlich and Krebs-2 carcinomas was intermediate and lay roughly in the order of their radiosensitivities. Anomalously, the C3HBA adenocarcinoma, which has low sensitivity and curability, showed a very marked response to concomitant systemic exposure.

In general, then, it may be concluded that the degree of enhancement obtainable relates to the radiosensitivity and radiocurability of the tumor. However, since various other characteristics of these tumors, such as extent of differentiation and growth rate, also follow in the same order as the sensitivity, it is impossible to state exactly which of these factors, if any, actually control the response to whole-body irradiation.

The artifacts possible with the use of transplanted rather than spontaneous tumors for irradiation experiments have recently received considerable attention. These factors must be considered in the interpretation of the present experiments, since the whole-body irradiation might be expected to have an effect on the immune mechanisms of the host animal. It is believed that such considerations may be largely negated. This is based on the assumption that any reaction of the host against the tumor transplant would be already in progress at the time of irradiation. From what is known about the time relations of the effects of irradiation on the immune process (*5*), it would not be expected that irradiation of the established tumor would have an effect. Beyond these time relations, it would not be expected that the total-body irradiation doses used in these experiments, especially in the lower range, would measurably depress the immune mechanisms of the mice. Furthermore, if any such depression of immune response were occurring, it would be expected that the systemic irradiation would tend toward decreasing the effect of the local irradiation on the tumor. This would be especially prominent in those tumors transplanted homologously as compared to those transplanted into the strain of origin. No such consistent loss of effect is seen with higher tumor doses. Also, there is no consistent relationship between the extent of enhancement obtained and whether the transplant is homologous or isologous. It is therefore likely that depression of the immune response plays little or no role in the results obtained in this study.

Among the mechanisms which may be proposed for this synergistic action of total-body and local irradiation is the possible influence of the endocrine system. The most likely of these glands, the adrenal, has been largely ruled out by the study of Hollcroft and Matthews (*6*) in which enhancement was still found in adrenalectomized animals.

There is some evidence that the immune process is enhanced by small doses of radiation (*5*), possibly through stimulation of the reticuloendothelial system. The presumption has been made that ingestion of small quantities of lymphocytes which have been destroyed by irradiation may have stimulatory effect on the macrophages. There is no evidence to indicate whether such a process is occurring in the present instance. If it were so, this would probably be more prominent in homologous than isologous transplanted tumors, as previously discussed. Since we did not find this true, this mechanism probably does not serve as an adequate explanation of our results.

Inferential evidence from the work of Hollcroft *et al.* (*2*) indicates that this potentiation is related to some unidentified but short-lived, blood-borne substance. Experiments in this laboratory (*4*) in which plasma from previously irradiated animals was injected concurrently with a local irradiation exposure provide direct evidence of such a mechanism and/or substance. Further experiments are in progress to define further the nature of this material.

To the extent that the synergistic effect of whole-body irradiation is mediated through its activity on the host, the factor of toxicity due to such treatment may require consideration. Where such toxicity for the host becomes limiting it may be inadvisable to use combined irradiation therapy even though it is more inhibitory for the tumor on a total-dose basis.

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A and B Antigens of Tumors Arising From Urinary Epithelium [1]

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SUMMARY

Cells from tumors arising out of the urinary epithelium in subjects of blood groups A, B, and AB were analyzed by the mixed-cell agglutination technique for the presence of relevant antigens. Complete or partial loss of antigen was frequently observed. Correlation with other properties of the tumors revealed increased frequency of antigen loss particularly among the more pleomorphic, anaplastic, infiltrating, and rapidly fatal tumors, but exceptions were numerous. There appeared to be no connection between the age of the patient and loss of antigen. These findings are discussed in the light of theories relating antigens to somatic mutation, ploidy, and tissue structure.—J. Nat. Cancer Inst. 26: 1349–1365, 1961.

THE A and B antigens in man form one of the most thoroughly explored systems in immunology. They have been found to exist in many, but by no means all, cells of the body, most notably in erythrocytes, various epithelial layers (*1*, *2*), and also in a number of tumors (*3–5*).

Two forms of the antigens are distinguishable—an alcohol-soluble and a water-soluble form. Whether the latter is present is largely under the control of the secretor genes. In the tissue under consideration, the transitional epithelium of the urinary tract, it appears that the alcohol-soluble form is predominantly or solely concerned (*2*). In a previous article (*6*) it was noted that, whereas A- and B-positive individuals always exhibited the antigens on all cells of this epithelium, tumors arising therefrom often appeared partially or completely deficient in antigen. Furthermore, there seemed to be some correlation between, on the one hand, the extent of tumor spread and the loss of structural and cytological differentiation and, on the other hand, the degree of antigenic loss. But the correlation was not close and conspicuous exceptions were noted.

In the studies here presented these observations were extended to include a larger total number of cases analyzed; in particular, the history of the tumor both before and after the time of testing was recorded.

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[2] We are indebted to Mr. K. G. Moreman for the photomicrographs and to Dr. Sylvia D. Lawler for valuable criticism.

METHOD

Cell suspensions were obtained and tested by the mixed-cell agglutination (MCA) technique of Coombs (7), as already described (6). Tumors of transitional epithelium contain relatively little stroma in relation to the volume of cells present, and since they do not produce a secretion, the overwhelming bulk of such a tumor is composed of the malignant cells themselves. Tumors with extensive necrosis or infection, or incidences in which it was impossible to obtain malignant cell suspensions that were not diluted with normal or stromal cells, have been omitted.

A portion of the tumor, removed surgically or at autopsy, is first washed in Tyrode's solution or Parker's medium 199 to remove loose erythrocytes or other contaminating material and then placed on a clean glass surface. Any small clots of blood are picked off with forceps. A bloodless but fleshy area is then selected and gently scraped with the end of a glass slide. This detaches large numbers of tumor cells without disrupting the stroma (fig. 1).

The fragments thus obtained are taken up with a Pasteur pipette into Tyrode's solution and may be left for up to 72 hours at 4° C. They should be broken up into a fine suspension shortly before they are tested. This is best effected by repeated rapid aspiration into a syringe through a #1 bore needle. The ease with which the cells are detached from each other is rather variable and the suspension must be frequently checked by microscopy. The aim is a suspension of single intact cells, but usually a few small clumps of up to 5 or 6 cells remain. Continued aspiration may eventually separate these, but at the risk of stripping the cytoplasm off some of the cells already isolated. It is best, therefore, to stop just short of the "perfect" suspension. Cells may now be counted; for the MCA test a count of 500 to 1,000 cells/mm^3 is used.[3]

One drop of this suspension is mixed with 3 drops of antiserum (routine grouping serum with a titer of 1:128–1:256) in a 4 mm tube and incubated at room temperature for 2 hours. At the end of this time a sample of the supernatant serum is tested with erythrocytes to ensure that there is still an excess of antibody, and the tumor cells are then washed 3 times in Tyrode's solution. To the remaining 1 drop of cells is added 1 drop of a 2 to 3 percent suspension of red cells in Tyrode's solution which may include AB serum (1:100) to prevent adhesion of cells to glass surfaces. The mixture is tapped up fairly vigorously.

The best and most consistent procedure at this stage is to allow another 1½ hours for incubation. A short cut is to spin the cell mixture lightly after only 15 minutes and this, with normal epithelial cells and some tumors, will produce complete agglutination. For reliable quantitative results with tumors the longer incubation is essential.

Examination of the final mixed agglutinate must be made under the high-power microscope with or without phase contrast. Negative results

[3] An error occurs in a previous paper (6) in that all cell-concentration figures were inadvertently multiplied by a factor of 16. The usual cell concentration employed is around 625 per mm^3.

and control tubes are easily read; the epithelial cells lie free among the red cells and any in contact can be shown to be nonadherent by some movement of the suspending fluid (fig. 2).

Partially agglutinated cells (fig. 3) can usually be easily recognized, but complete agglutination may almost totally obscure the epithelial cell. The shape and size of the agglutinate and the presence of a dark, granular center, when phase contrast is used for focusing, are usually sufficient for recognition. If necessary, a small quantity of distilled water can be run in from the edge of the coverslip and this will lyse the erythrocytes and expose the cell at the center. False-positive agglutinates are caused by contamination of red cells from the original suspension, and the presence of a few of these is sometimes inevitable. Their size, shape, and density distinguish them from mixed agglutinates. Disintegrating cells may entrap some red cells probably among strands of deoxyribonucleic acid. These agglutinates can be distinguished by their shape and are also present in control tubes.

All positive tubes are assembled in duplicate, with negative controls as follows: for A epithelial cells, anti-A with B erythrocytes, anti-B with A erthyrocytes, and anti-B with B erythrocytes. The converse is used for B cells, while for AB cells O erythrocytes provide the negative controls.

A positive control for an antigen-negative tumor can be provided only if normal cells are simultaneously available. This does not always happen and one must then rely on the past consistency of the test.

In the assessment of the results of the MCA test, two parameters are theoretically available: the proportion of epithelial cells showing one or more adherent erythrocytes and a more complex measurement to include the number of erythrocytes adhering to each cell. With normal transitional epithelium, the surface of virtually 100 percent of the cells is completely covered with erythrocytes, and when some cells in tumors are found without adherent red cells, others are always discovered with only a partial covering. In practice, therefore, it is simpler to take only the first parameter, and, in all that follows, the terms "agglutination" or "agglutinability" will refer to this property. It must be remembered that in all intermediate categories many of the cells showing agglutination lack the full complement of indicator erythrocytes.

A rough count of 100 to 200 cells enables a classification to be made as follows (table 1):

TABLE 1.—Degree of agglutination*

Number (percent)	Symbol
90–100	+++
50–90	++
Approximately 50	+(+)
10–50	+
0–10	−

*That is, number of epithelial cells with one or more adherent red cells.

Accuracy is limited by the difficulty of ascertaining whether a particular clump contains a carcinoma cell or only a contaminant erythrocyte or clump of erythrocytes. A surprising degree of accuracy, however, is sometimes evident, and the middle intermediate category +(+) was introduced with great reluctance when a tumor twice tested refused to fall into either of the adjacent groups.

It is important to note two distinctions between the persistence of antigen demonstrable by the MCA test and fluorescent antibody staining. Szulman noted deterioration of staining properties within 3 to 6 hours in autopsy material, and Holborow *et al.* comment on the better results from fresh tissues. By contrast, the MCA test performed on ureteric epithelium, *i.e.*, normal control cells, usually from autopsies and obtained up to 48 hours after death, has shown no such deterioration. Furthermore, whereas the basal layer of the transitional epithelium shows little or no staining, it clearly bears antigens when tested by mixed-cell agglutination, since 100 percent of the cells react in this test. Complete denudation down to the basement membrane has been checked by histological section.

Obviously the MCA test is the more sensitive measure of the presence of antigen and, since the tests on tumor cells have revealed a diminution of antigen in the majority of cases, the test is on the whole preferable. Loss of antigen with this test reveals a definite abnormality so that the differences found cannot be explained in terms of the cell layer from which the tumor arose, though this may be a factor.

Histological examination was confined to paraffin sections stained with hematoxylin and eosin.

RESULTS

Samples from 60 patients, including the 25 previously reported (*6*), on whom histological details were available, are analyzed in table 2. Forty-two control normal suspensions were also tested, the cells being derived from both secretor and nonsecretor, adult and fetal epithelium of ureter and bladder.

It was possible to test both primary tumor and metastases only 3 times: twice simultaneously; in one patient at operation and 19 months later at autopsy. Different parts of the primary tumor were often tested both simultaneously, or at biopsy and subsequent cystectomy, and in 3 instances metastases from different organs of the body were analyzed. In all these multiple tests no difference was noted in the degree of agglutinability of the tumor cells. In one instance, however, a tumor showing an incomplete (++) agglutination recurred or a new tumor arose (22 months later) showing complete agglutination (+++). Unfortunately, no histological examination was made on the second occasion.

The relationship between agglutinability and three properties of the tumor is shown in text-figures 1, 2, and 3.

CARCINOMA OF BLADDER: MCA TEST: A and B ANTIGENS

Cytology	Degree of Agglutination: 0	+	+(+)	++	+++	Total
Uniform		4	1	7		
	4½		13		10½	27
Moderately Variable		3	1½	3		
	7½		6½		4	18
Pleomorphic		0	0	4		
	5		4		6	15
	17		23½		20½	

TEXT-FIGURE 1.—Relationship between agglutinability and histological structure of tumors.

CARCINOMA OF BLADDER: MCA TEST: A and B ANTIGENS

Structure	Degree of Agglutination: 0	+	+(+)	++	+++	Total
Well-differentiated		3	1	7		
	4½		11		11½	27
Poorly differentiated		0	1½	3		
	7½		4½		4	16
Anaplastic		3	0	4		
	5		7		5	17
	17		23½		20½	

TEXT-FIGURE 2.—Relationship between agglutinability and cytological characteristics of tumors.

CARCINOMA OF BLADDER: MCA TEST: A and B ANTIGENS

Spread	Degree of Agglutination: 0	+	+(+)	++	+++	Total
Superficial		0	1½	7		
	5		8½		8½	22
Muscle lymphatics		3	1	6		
	5		10		9	24
Metastases		3	0	1		
	7		4		3	14
	17		22½		20½	

TEXT-FIGURE 3.—Relationship between agglutinability and established degree of spread of tumors.

Classification of the tumors was made from sections: Histological structure was divided into categories of well-differentiated, poorly differentiated, and anaplastic; cytologically they were divided into uniform, moderately variable, and pleomorphic. In the latter, particular attention was given the nucleus and the frequency of obviously polyploid cells.

TABLE 2.—Analysis of samples of tumor tested from 60 patients

	Tumors of bladder epithelium	Transitional-cell tumors of kidney
Primary tumor only	48	2
Metastases only	7	0
Primary and metastases	2	1
Operation specimen only	50	2
Autopsy specimen only	5	1
Operation and autopsy specimens	2	0
Group A	46	2
Group B	9	1
Group AB	2	0
Total	57	3

In general, the degree of deviation from normal in these properties tends to coincide but, as will become clear in the discussion, the distinction is potentially important.

In only two instances was it difficult to assign tumors to a particular category, and in these there was such variability—some well-differentiated with uniform cells, some poorly differentiated with moderate pleomorphism—that these two cases were placed half and half in the first two categories. This is obviously a compromise solution for, although it is probable that the cell suspensions tested contained cells from both types of tumor patterns, this is not necessarily true. Unfortunately in these cases only a single suspension was made from the tumor. One other result indicated by "halves" concerns a tumor from a group A_1B subject in which the B antigen was normally expressed, the A antigen only partially so. This was confirmed by a retest.

In text-figure 3 the tumors have been divided into three categories according to the apparent degree of infiltration or presence of metastases at the time of testing.

In the superficial group the tumor is limited to the mucosa and submucosa; in the second group there is infiltration of bladder musculature and/or lymphatics, while in the third group metastases are in lymph nodes or distant organs.

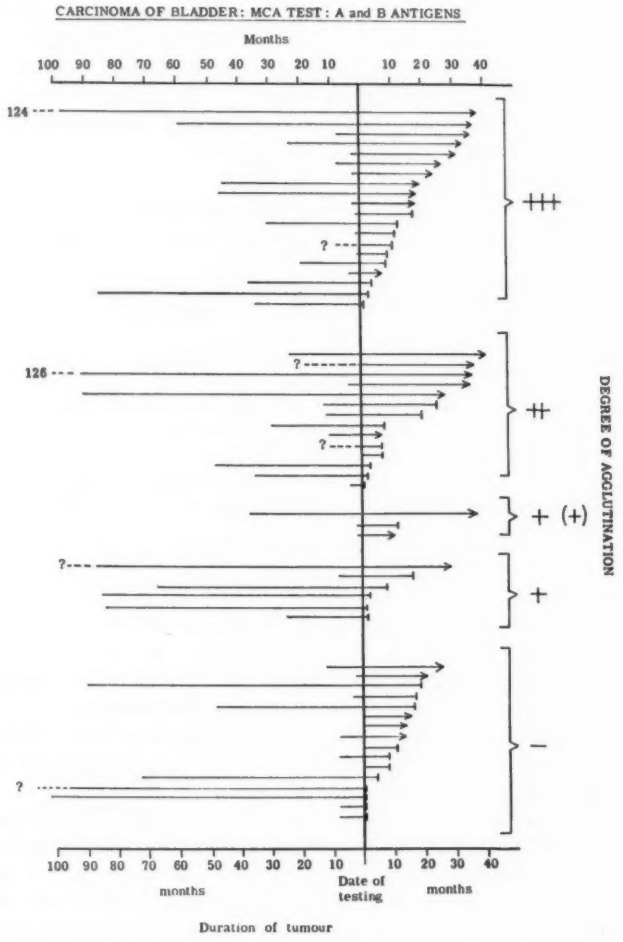
In every case, except one, this distinction is based on histological examination of an adequate sample of the tumor, with an adjacent portion of bladder wall or kidney. In the one exception biopsy was inadequate, but it was evident at operation that widespread infiltration of the bladder musculature had occurred.

It is evident, of course, that the degree of infiltration so shown is the least that may have occurred and that infiltration outside the biopsy area or unseen distant metastases may have been present.

Only the future course of the disease provides any insight into the actual malignant potential of the tumor cells and even this is subject to

modification as a result of treatment. The latter has been carried out by standard methods as described by Wallace (*8*), and, since the actual form of treatment has not beeen influenced by the revealed antigen content of the cells, a follow-up of the patients is a valid indication of the malignancy of the tumor.

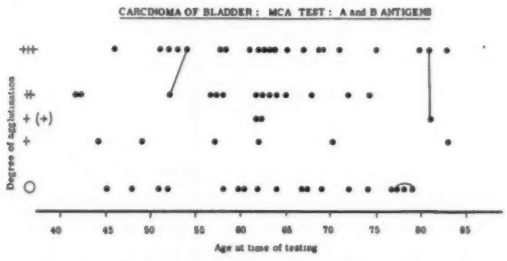
Fifty-seven patients have been followed for periods up to 40 months from the time of testing. The results are shown schematically in text-figure 4 in which the central line indicates the moment of testing. The



TEXT-FIGURE 4.—Relation of agglutination to previous history of tumor and subsequent progress; ⊣ indicates date of death; → indicates patient alive at date of last follow-up.

time before testing, which was occupied by tumor-indicative history and, in many cases, by previous treatments, is indicated at the left. To the right the follow-up period of variable length is shown, which also reveals whether the patient has died or is still living. Thus any connection between the antigen content and the future behavior of the tumor or between loss of antigen and total tumor duration can be seen. Such a connection would be expected if the phenomenon of "progression" occurs at all frequently in human bladder tumors.[4]

Finally, text-figure 5 shows the age of each patient at the time of testing and the degree of agglutination. These data constitute a test of the postulate that the frequency of an observed change of phenotype is proportional to the time during which such a change, possibly a somatic mutation, could have occurred. The somewhat higher incidence of well-differentiated tumors in the younger age groups (*9*) might tend to accentuate the degree of correlation if any were found, but it is clear that the distribution of points is completely at random.



TEXT-FIGURE 5.—Relation of agglutination to age at time of testing. Communicating lines indicate tests on same patient, on 2 patients on different dates, on 1 patient (AB) A and B antigens simultaneously.

DISCUSSION

From these results it can be assumed that no close correlation exists between antigen content and other aspects of tumor behavior. In text-figures 1, 2, and 3 it is easier to consider the figures in each corner, where one might expect that those in the lower-left and upper-right corners would exceed those in the opposite position. This is found to be true, but the excess is small and evidently due to the presence of a number of well-differentiated, noninfiltrating tumors of uniform cells with normal antigenic characteristics. With these tumors disregarded, it is apparent that tumors may totally lose their antigens while remaining relatively benign and, conversely, that anaplastic pleomorphic tumors with metastases may have normal A and B antigens.

[4] In this context one speaks of "a tumor" which perhaps is treated and recurs, though in many instances the probability is that a new tumor has arisen.

Högman (*10*) has reported that cultures of kidney cells regularly lose their AB antigens, as detected by mixed-cell agglutination, after a number of subcultures. On the other hand, the normal transitional epithelium is rather constant in its preservation of antigen. One must assume, therefore, that in one or the other of these situations, possibly both, some selective pressure exists to establish and maintain conformity of cell type, but this pressure may not be directly exerted for or against the antigen itself. This factor is evidently a variable in neoplasia and, as such, it is difficult to see how it is related in any direct way to the process of carcinogenesis. If, however, it is true that some alteration of the genome is an integral part of carcinogenesis, then knowledge of changes at various exactly defined points of the gene complex (including perhaps plasmagenes) might enable one eventually to estimate the number, size, and even the position of those changes essential to carcinogenesis.

The ABO complex provides in many ways a useful system in this respect. If we exclude the rare suppressor genes, such as those responsible for the "Bombay bloods," which even in heterozygous form must be excessively rare but which presumably have active alleles, the expression of AB antigen may be dependent on genes at two known loci—*i.e.*, the secretor genes that determine the production of water-soluble antigen and the ABO genes themselves—as well as on other less well-defined influences. Examples of the latter occur in fetal life (*11–13*) and probably also in rare cases of leukemia (*14, 15*) in which the erythrocyte antigens may be partly or completely lost. Preliminary studies tend to indicate that neither secretor status nor, in the group A subjects, the subgroup of A (A_1, A_2, etc.) has influence on the transitional epithelium whether normal (*1, 2*) or neoplastic. But the difficulty of distinguishing between change of genotype and phenotype remains.

Hauschka (*16, 17*) has postulated that tumors with a normal diploid or near-diploid mode of chromosomes retain the antigenic properties of the cells of origin and, being incapable of antigenic modification, are not transplantable outside the strain of animal in which they arise. By contrast, heteroploid tumors, *e.g.*, with hyperdiploid, tetraploid and near-tetraploid modes, can by "antigenic simplification" adapt to the adverse environments encountered on transplantation to a foreign strain.

Although no karyotypic analyses have been undertaken in the tumors here reported, some useful guesses can be made of the degree of heteroploidy from estimates of the size and variation of cell nuclei. This is represented in table 2 and, as already remarked, there are several exceptions to the expected relationship. Figure 4, for example, illustrates a pleomorphic tumor which gave 100 percent response with anti-A in the MCA test. The tumor in figure 5, by contrast, gave a totally negative result. Such tumors of both types have now been observed with sufficient frequency to place their existence beyond doubt.

It seems probable, therefore, that there is no direct relationship between the antigenicity of these "spontaneous" human tumors and the degree of nuclear pleomorphism (which is an expression of heteroploidy).

It is, perhaps, misleading to compare such tumors with those of laboratory mice in which the artificial circumstances of transplantation exert selective pressure, but no other standard for comparison exists. It is probably reasonable to suppose that any tumor represents a most successful clone arising from competition under more or less adverse conditions. A series of tumors, therefore, will include cells with ranges of adaptation, all greater than the normal, but differing from tumor to tumor. At present it seems likely that both heteroploidy and loss of antigenicity are very loosely linked and perhaps indirect expressions of this adaptive capacity, rather than being either its cause or causally interrelated.

If a tumor with a wide adaptive range is more likely to shed antigens than one less versatile, it is also more likely to spread quickly and widely and to survive better the onslaughts of various forms of treatment. A study of text-figure 4 tends to confirm this view. Omitting the 6 cases tested only at autopsy, one can see at a glance that the antigen-negative group carry the worst prognosis and that, very approximately, the nearer the agglutination reaction to normal the greater the probability of survival of the patient.

The same figure reveals very little evidence for the existence of "progression" (*18*) of tumors in this series. If gradual progression from benign to malignant were at all frequent, one would expect that the pretesting duration would be greater in the antigen-negative than in the antigen-positive groups, since the former would include those progressed tumors which have shifted from positive to negative. In fact this period in most antigen-negative tumors is short [in 11 of 16 less than 1 year compared with 10 of 20 in the positive (+++) group], but this does not exclude the possibility that some tumors may change their characteristics. More work evidently remains to be done in serial testing before any firm conclusion can be reached.

The question is often asked, "What purpose is served by transplantation antigens and antibodies in nature?" (*19*). One answer, which has come to be known as the Tyler-Weiss hypothesis (*20*, *21*) is that the specific chemical groupings on cell surfaces help to maintain cell adhesion in tissues and influence differentiation. An experiment quoted in support of this theory is that of Spiegel (*22*), in which dissociated sponge cells were inhibited from reaggregation by specific antisera but, as Spiegel himself admits, this finding will support more than one explanation. The spontaneous loss of antigen observed in neoplasia might, if reflected in the altered structure of the tissue, form a more useful test system. The relevance of the present observations depends upon one's view of the ABH antigens as typical cell-structure antigens. Their widespread occurrence in high concentration on the surface of many epithelial cells might tempt one to this belief, while such cogent arguments as the presence of ABH on essentially detached units, *e.g.*, blood cells, will oppose it. One may, however, point to instances (fig. 6) in which structure is lost but antigens are not, and others (fig. 7) in which the opposite occurs, and so draw the appropriate conclusions.

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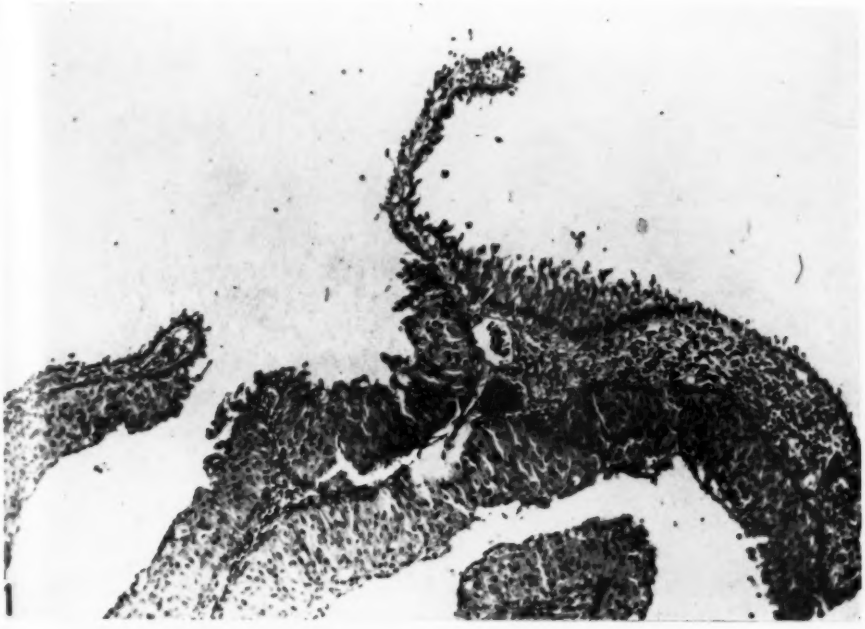
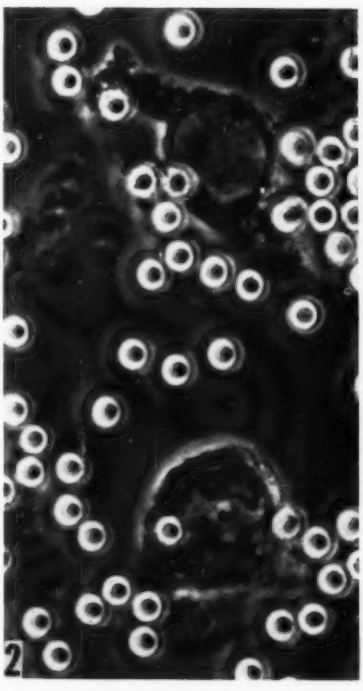
PLATE 198

FIGURE 1.—Carcinoma of bladder. Tumor cells have been scraped from surface of tumor without injury to stroma.

FIGURE 2.—Mixed-cell agglutination. Negative result. Two tumor cells lie free among indicator erythrocytes.

FIGURE 3.—Mixed-cell agglutination. Positive result. Small clump of tumor cells have adherent erythrocytes on their surface. Many erythrocytes are consequently outside plane of focus.





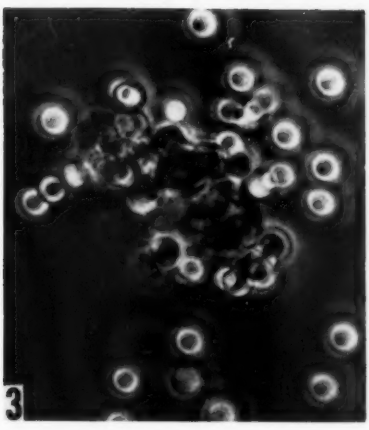
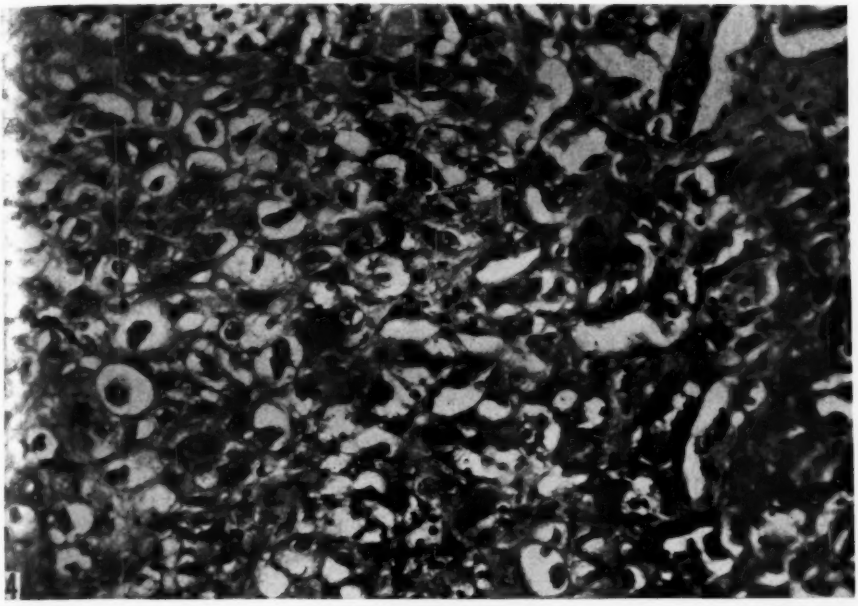


PLATE 199

FIGURE 4.—Pleomorphic carcinoma of bladder; group A. MCA test: +++. × 320

FIGURE 5.—Well-differentiated carcinoma of bladder. Uniform cells; group A. MCA test: −ve. × 320



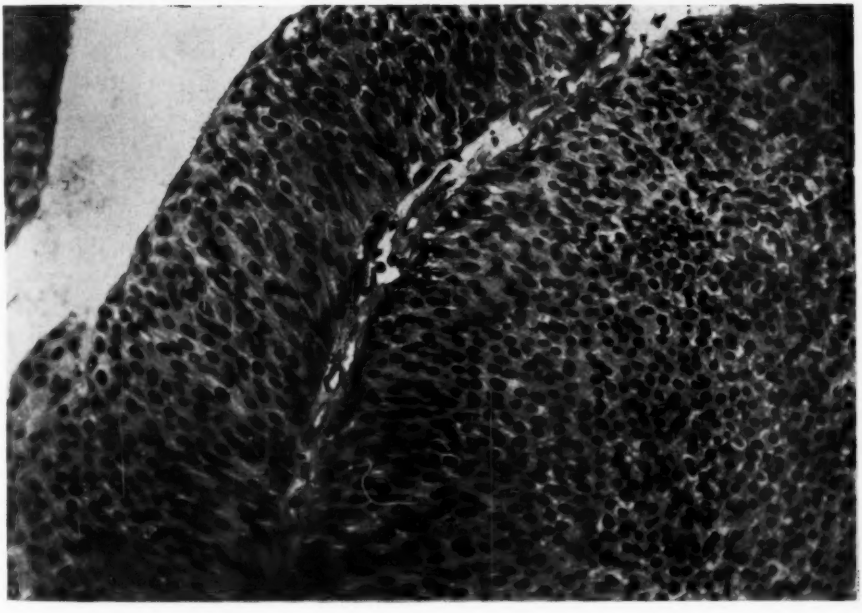
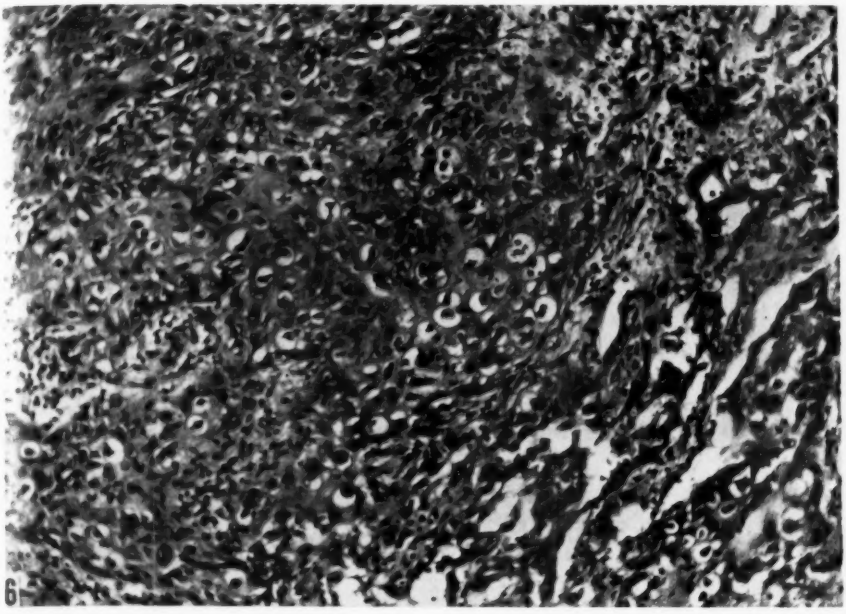


PLATE 200

FIGURE 6.—Anaplastic carcinoma of bladder; group A. MCA test: +++. × 160

FIGURE 7.—Well-differentiated carcinoma of bladder; group A. MCA test: −ve. × 160



Host Factors in Cell Culture: Further Studies on the Growth-Controlling Action of Fresh Human Serum [1, 2]

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SUMMARY

This study confirms and further elucidates the previous observation that permanent differences exist between fresh human serums in their growth-controlling capacity. In fresh human serum, there were factors which prevented cells in cell culture from growing in a loose migratory structure. These factors caused cell clumping and reduced cell viability. The nuclear size of cells growing in a clumping serum was smaller, and there were fewer cells with high chromosome counts. Serums which caused strong clumping of cells in cell culture were relatively rare. No connection with blood groups was observed. Since cell growth *in vitro* differs markedly in fresh serums obtained from different persons, the possibility is discussed that the growth of cells—and cancer cells—could also *in vivo* be different in these persons. Preliminary observations indicated that these factors could be connected with low-density proteins.—J. Nat. Cancer Inst. 26: 1367–1379, 1961.

IN EACH human life there probably are stimuli and opportunities for cancerous transformation of some cells. The progress of these cells to the clinical tumor stage could be due to a deficiency in the growth-controlling mechanism of the host. To demonstrate the existence of possible factors which control growth, since 1955 we have studied the effect of human serum on different cells in cell cultures (*1*, *2*). In these studies, we observed that permanent differences exist among fresh human serums in their growth-promoting capacity in cell cultures; these differences were noted by examination of the mode of growth, multiplication rate, size of nuclei, chromosome counts, and the viability of cells (*3*, *4*). In some human serums, the cells grow in dense clumps and not in the more common loose migratory structure. Repeated feedings with such fresh serum can even kill cell cultures. The nuclear size in cell cultures grown in these serums is smaller, and chromosomal studies have shown that more cells are seen with an exceptionally low chromosome number. Repeated

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[3] The authors wish to thank Miss Dyweke Bremer, Miss Ilta-Meri Santaoja, and Mr. P. Korhonen for their technical assistance.

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bleedings from the same individuals during a period of more than 5 years revealed stability in the mode of growth in fresh serum taken from the same individual.

This paper presents some new general observations on the growth-controlling effect of fresh, normal human serum.

MATERIAL AND METHODS

Cells.—Two HeLa cell lines, the Detroit-6 cell line, a continuous amniotic cell line (Utrecht), and primary amnion cell cultures were used.

Collection of serum specimens.—Blood from a blood bank, and other sources was used. In no instance was anticoagulant present. The tubings used in the blood bank were specially tested for possible toxicity. Individual samples were collected in vacutainers. The clot was not allowed to retract for more than 24 hours and generally for a shorter period. If not immediately tested, the serum collected after centrifugation was stored in a −70° C deep-freeze.

Cell culture mediums.—Unless otherwise stated, the results are those obtained with HeLa cells that had been growing continuously in our laboratory since 1955 in 30 percent heat-inactivated (56° C), Seitz-filtered (pressure), human serum in Hanks' solution. In some series, cells grown during several passages in unfiltered serum pools were also used, with no observable difference in the results. Streptomycin (50γ/ml) and, earlier, penicillin (50 U/ml) were used routinely in maintaining the stock cultures and in the experiments. Difco-trypsin, 1:250, was used as 1 percent stock solution. The solution was sterilized by Seitz filtration. According to tryptic-activity determinations, some of its activity was lost in this process. For additional safety in *p*H control in the experiment, 0.16/M tris buffer, *p*H 7.2 (Sigma 7–9), was used, as described later.

Setup of experiments.—HeLa cells grown in Roux bottles for 2 to 3 days after seeding were trypsinized by use of a calculated final concentration of 0.15 percent of the trypsin preparation in Hanks' solution. The cells were trypsinized for half an hour at 36° C. The dispersed cells were then centrifuged for 10 minutes at 1000 rpm., the supernatant was discarded and the cells were suspended in Hanks' solution. After the cells were counted in a hemocytometer, the cell dilution was corrected to represent 20 to 40 thousand cells per inoculum of 0.65 ml.

The order of pipetting the test tubes (16 × 120 mm) was as follows: 1) 0.05 ml of tris buffer, 2) 0.3 ml of serum or serum fraction, and 3) 0.65 ml of cell suspension. The tris buffer and serum or serum fraction to be tested were allowed contact for 10 minutes before addition of the cell suspension. The tubes had rubber stoppers ("Grave" Stockholm), and the rubber was tested for toxicity during the experiments. No toxicity for cells was ever observed.

Controls.—In all the series, a known old serum pool and a known "clumping" serum were included. These controls were established to

show that the cells were in proper condition after trypsinization and that they were properly dispersed without initial agglutination, which occasionally occurs. At the same time it could be determined whether the typical clumping phenomenon occurred with the known clumping serum.

Phenol red in Hanks' solution served as *p*H control of the individual tubes in the experiment. No differences were observed between the tubes when the technique previously described was followed. In the most toxic fractions the *p*H turned to the alkaline side—not immediately, but after some hours of incubation, and this was obviously due to cell death and cell disintegration.

A modification of the citric-acid method (*5*) was used in counting the nuclei. The nuclear size was measured from stained smears after citric-acid treatment and directly from stained coverslips. The images of nuclei were drawn on paper (1,000×) and the areas measured by planimeter. The chromosomes were counted microscopically (1,200×) and 100 cells were counted from each cell line. The method was as follows (*6*): 18 hours in Colcemide (Ciba) concentration of 10^{-6}, osmotic expansion, fixation, hydrolysis according to Tjio and Puck (*7*), and staining with Feulgen for nuclear reaction.

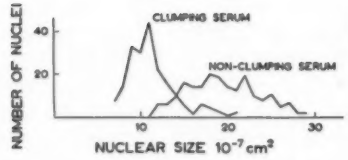
Centrifugation.—The Spinco ultracentrifuge Model L with rotor #40 was used in some experiments. The serum was transferred by pipette into 10 cc lusteroid tubes and centrifuged for 5 hours at 38,000 rpm. The average centrifugal force was thus of the order of 100,000 × *g*. The different serum fractions were collected with a syringe and needle, and special care was taken to avoid blurring. To obtain the usual serum fractions, cold-alcohol and ammonium-sulfate precipitation methods were used. Ultracellafilter "fein" was used in ultrafiltration experiments. The blood samples were obtained through the courtesy of H. R. Nevanlinna, M.D., Director, Finnish Red Cross Blood Bank.

RESULTS

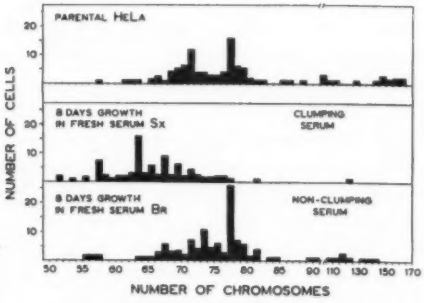
Clumping Phenomenon

In some normal fresh serums the cells grew in dense, compact colonies, and not in the usual loose migratory structure. Individual cells in the clumps were difficult to discern at low magnification, and the glass surface between the clumps was almost free of cells (fig. 1). It was observed by cinematography that some cells in the clumps adhered to the glass surface, and that the rest of the clumps pendulated in the fluid. The dividing cells remained in the clumps and thus continued to become increasingly compact. Furthermore, some rapidly disintegrating cells were often seen when clumping serum was used as a growth medium. The growth behavior of cells in clumping serum, in comparison with that of cells in "old" serum and serum with no clumping effect, is illustrated in the series of pictures from time-lapse cinematography shown in figure 2.

In serums which cause strong clumping of cells, the nuclear size is smaller and fewer cells have an exceptionally high number of chromosomes (text-figs. 1 and 2). The multiplication rate and growth, as measured by the citric-acid method and cellular-nitrogen determinations, are slower in clumping serum than in nonclumping serum (text-fig. 3).



TEXT-FIGURE 1.—Distribution of nuclear size after 2 days' growth in clumping and nonclumping serum.


NUMBER OF CELLS
PARENTAL HELA
8 DAYS GROWTH IN FRESH SERUM Sx
CLUMPING SERUM
8 DAYS GROWTH IN FRESH SERUM Br
NON-CLUMPING SERUM
20
10
50 55 60 65 70 75 80 85 90 110 130 150 170
NUMBER OF CHROMOSOMES

TEXT-FIGURE 2.—Chromosome distribution in the parental HeLa cell line after 8 days of growth in fresh clumping and nonclumping serum.

Nonclumping serum did not cause cell clumping, even when used as 90 percent dilution in Hanks' solution, but strongly clumping serum caused clumping of cells in 5 percent dilution in Hanks' solution. This clumping phenomenon also appeared when the other cell lines mentioned were used.

Frequency of Different Serum Types in Regard to Clumping of Cells

Serums which cause strong clumping of cells in cell culture are relatively rare, as are totally negative, *i.e.*, nonclumping serums. Of 290 serum samples studied the past year, strong clumping was observed in 26 samples and no clumping whatsoever in 17. Thus 9 percent of the serums caused strong clumping in a series of healthy blood donors, and an even smaller percentage of totally negative, *i.e.*, nonclumping serums. Most serums belonged to the intermediate group. Many of the serums of the 2 extreme groups were studied as many as 25 times—always after a new bleeding. In these experiments, growth behavior was constant in the same serum.

Connection With Blood Groups

The familiar occurrence of serums that cause strong clumping, which we reported earlier (*3*), led us to study blood groups. Table 1 shows the frequency of ABO blood groups among donors of different serum types, and table 2 shows a further distribution of the blood groups in 5 of the persons studied.

TABLE 1.—Distribution of ABO blood groups in persons with clumping or nonclumping serum

Serum	Number of cases			
	Total	A	B	O
Clumping	26	14	2	10
Nonclumping	17	7	3	7

TABLE 2.—Further distribution of blood groups in 5 persons with clumping or nonclumping serum

Serum	Blood group	M	N	C	D	E	c	P
Clumping	O	+	−	+	+	−	+	−
Clumping	A	+	−	−	+	+	+	−
Clumping	A_1	+	−	−	+	+	+	+
Nonclumping	A_1	+	−	+	+	+	+	−
Nonclumping	O	+	+	−	−	−	+	+

According to these tables, no correlation exists between the growth-controlling (clumping) effect of serum and the blood groups studied. The haptoglobulin group was also the same (2–1) in our two types of serums for strong clumping and nonclumping.

Instability of Growth-Controlling System *In Vitro*

The clumping effect could be observed only when the serum was fresh. All old serums, *i.e.*, those which have been stored at room temperature or in a refrigerator for some time, were negative (nonclumping). Their growth-promoting capacity was very good and usually better than that of fresh or heat-inactivated serum. Normally the clumping effect was destroyed or weakened by heating serum above 52° C for 30 minutes. The growth-promoting capacity of clumping and nonclumping serums were adversely affected by heating. Heat inactivation of clumping serum increased its growth-promoting capacity, whereas in nonclumping the effect, if any, was usually the reverse. Table 3 shows the effect of inactivation on the nuclear counts in two serums, one clumping the other nonclumping, which were studied repeatedly—always after a new bleeding.

TABLE 3.—Data on effect of inactivation on nuclear count of one clumping and one nonclumping serum, after repeated study—always after a new bleeding*

Serum	Number of experiments	Higher cell count after inactivation	Lower cell count after inactivation	No difference in cell count after inactivation†
Clumping	25	13	1	11
Nonclumping	20	2	8	10

* The arithmetical mean of 2-day counts from 3 tubes was used as a basis for comparison.
† No difference in cell count means that the difference was below 10 percent.

Fractionation of Serum

Conventional methods for the fractionation of serum have failed due to lability of the factors that cause clumping. In our experience the fractions, after ammonium-sulfate and alcohol precipitation at low temperatures, have often been toxic. No fractions that cause the clumping of cells have been obtained by these methods. The only technique which has resulted in clumping fractions has been ultracentrifugation.

After 5 hours' ultracentrifugation by the method described, in all fresh serum there was a whitish supernatant with a sharp boundary to the rest of the serum. This formed some 10 percent of the total volume; its protein content varied between 0.7 and 1.2 percent. This fraction, I, caused strong clumping of cells, even when the whole serum did not have this effect. The cells seemed severely damaged and did not survive more than 2 or 3 days. The clumping effect of this fraction could not be destroyed by heating (56° C, 30 min) or by storage for several weeks in a refrigerator. A fraction similar to that obtained from fresh serum was also secured from heat-inactivated serum, but usually not from "old" serums. When the rest of the centrifuged serum was recentrifuged, a fraction similar to fraction I was obtained; this procedure can be repeated at least 3 times. The clumping effect of fraction I disappeared rapidly when nonclumping serum or serum fraction was added to it. However, this process of "inactivation" apparently was not instantaneous. The flattening fraction obtained by Lieberman and Ove's method (*8*) had, in this respect, the same effect as whole nonclumping serum.

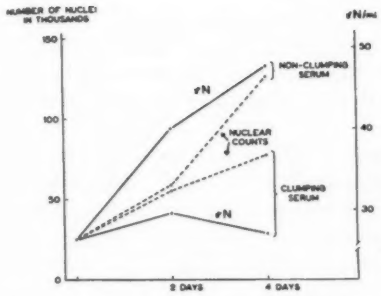
The middle fraction, II, was aspirated to form 70 percent of the serum. It had the usual color of serum and its protein content varied from 6.5 to 7.5 percent. The growth-promoting capacity of this layer was very good. This fraction caused clumping of cells only when clumping occurred in the original whole serum. In contrast to fraction I, the clumping effect could be destroyed by heating.

The rest of the serum, the bottom layer, was termed fraction III. It had a protein content of 10 to 11 percent. This fraction caused partial clumping of cells when the whole serum caused strong clumping, but not otherwise. A slight granulation of the cells was often observed when fraction III was used but its growth-promoting capacity was in general good.

If fraction I was recentrifuged, 3 fractions could again be obtained. The effect of different fractions of serum are schematically presented in text-figure 4.

Preliminary Studies on Some Physical Properties of Clumping-Causing Supernatant Fractions

When fraction I, which caused the strongest clumping, was dialyzed, it was observed that the factors that caused clumping did not penetrate the membrane. This same observation had also been made earlier with whole serum.



TEXT-FIGURE 3.—Growth rate of HeLa cells in clumping and nonclumping serum, as measured by the counting of nuclei and determination of cellular nitrogen.

Ultracentrifugal fractions	Clumping serum		Non-clumping serum	
	fresh	inactivated	fresh	inactivated
Fraction I	+++	+++	++	+
fraction I_1	0	0	0	0
fraction I_2	++	+	–	–
fraction I_3	++	–	–	–
Fraction II	++	–	–	–
Fraction III	+	–	–	–

TEXT-FIGURE 4.—Degree of clumping in different serum fractions with clumping and nonclumping serum. 0 = all cells killed; +++ = very strong clumping; ++ = strong clumping; + = clumping; – = no clumping.

In ultrafiltration experiments with ultracellafilter "fein," it was observed that the filtrate did not have properties that caused clumping, but the large molecular part that washed out from the filter caused cell clumping.

Paper-electrophoretic studies indicated that this fraction contained larger amounts of certain lipoproteins when it came from clumping serum than when it derived from nonclumping serum.

DISCUSSION

The observation previously reported by us, that permanent differences exist between fresh human serums in their growth-controlling capacity, have been further studied. It has been shown that in fresh human serum there are factors which prevent cells in cell culture from growing in a loose migratory structure. These factors cause clumping of cells and reduce their viability. The nuclear size of cells growing in such serum is smaller and fewer cells with high chromosome counts are observed.

Since homologous serum and cells were used, it is difficult to compare these results with other observations in which heterologous serum or cells were used (*9*, *10*).

Human serums that cause strong clumping of cells in cell culture are relatively rare among blood donors (about 9%), as are also serums with no clumping effect. Most serums belong to an intermediate group.

The clumping phenomenon described in this paper can be observed only if the serum is fresh. A given amount of serum can change the behavior of only a limited number of cells and only for a limited time. Thus, if the inoculum size is too big, or the serum has been stored at room temperature or refrigerated for some days, no effect, or only a slight one, can be seen. This agrees with our earlier observation (*11*) that absorption of the serum with HeLa cells removes or weakens the clumping effect.

The observation that "old" serum has good growth-promoting capacity which is better than that of fresh serum cannot be explained only by degradation of serum proteins and the release of growth-promoting factors (*12–16*), since a decrease in the growth-controlling factors must also be expected.

The reduction in viability of the cells mentioned is more pronounced if the cell culture is repeatedly refed with fresh serum and if the cells are at the same time trypsinized (*17*). The effect of trypsin may indicate that some uncoating of the cell from surface proteins makes it more susceptible to the effect of the serum factors. This is consistent with the hypothesis that the "protein bound to the cell is in itself a surface, and as such may regulate surface dependent mechanisms important for normal cell physiology." (*18*).

When we tried to analyze the growth-controlling factors in more detail they seemed to disappear and it was difficult to isolate and identify them. The only method with which we have achieved some success is ultracentrifugation. By this means a supernatant is obtained which causes very strong cell clumping. Such a fraction, though less potent, can also be obtained from nonclumping serum. As mentioned previously, this fraction, when it comes from clumping serum, causes a strong clumping of cells, and is appreciably stronger than that attained with whole serum. All cells are usually killed within 2 to 3 days, though the protein content, as such, is sufficient for maintaining viability of the cells. From this supernatant, a still more potent fraction can be obtained by repeated centrifugation and then collection of the supernatant. The behavior of cells in the other two centrifugal fractions is more like that in the whole serum.

There is no conclusive proof that in this supernatant fraction the factors are those that cause clumping of cells when whole serum is used, though the effect of this fraction is stronger when it derives from clumping serum than when it comes from nonclumping serum. The fact that the effect of this isolated clumping-causing supernatant fraction disappears with relative rapidity after the addition of other serum proteins may indicate the existence of antifactors against clumping. This could explain why a clumping fraction can also be obtained from nonclumping serum.

These discussions agree with the more general hypothesis that an equilibrium exists in an organism between the production and destruction of growth-controlling and growth-promoting factors.

Since cell growth *in vitro* in fresh serums can vary so markedly between different individuals, as indicated in this paper, the possibility that the growth of cells, and cancer cells, could also be different *in vivo* in those persons must have serious consideration. The clinical investigation and follow-up examination of persons belonging to the two extreme groups could reveal the importance, or uselessness, of our grouping of individuals by clumping phenomena. The observation that serum from cancer patients has a good growth-promoting effect (*19, 20*) is interesting, as is

the fact that more marked and more prolonged growth of implanted cells occurs in cancer patients than in normal persons (*21*).

A question that must be studied further is the connection between our observations on fresh human serum and the recent observations on cytolytic serum factors (*22*).

The nature of the growth-controlling factors in serum is still unsolved. Nevertheless, the results obtained with ultracentrifugation indicate that the density of these factors is low. The behavior in dialysis and ultrafiltration experiments points to low-density proteins. Preliminary paper-electrophoretic studies of clumping and nonclumping serums and their different fractions seem to indicate that certain lipoproteins are present in major amounts in serums and in fractions causing strong clumping than are in those without this effect. This agrees with the observation that extreme values in fatty acid and lipide-P fractions of serum seem to be correlated with lower growth-promoting properties or toxicity (*23*).

The growth-inhibiting principles in serum were correlated with the lipide fractions of serum by Carrel and Ebeling as early as 1922 (*24*).

More studies with fresh human serum are needed. Experiments with autologous cells and serums would be most interesting.

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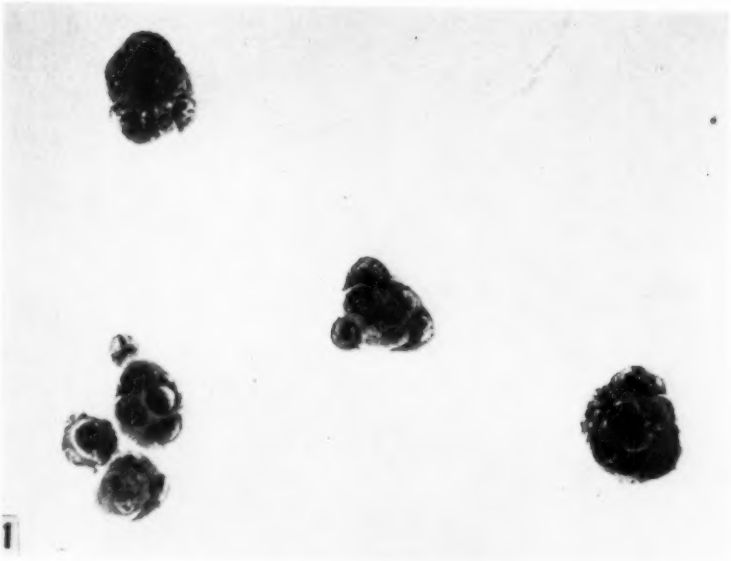
PLATE 201

FIGURE 1.—Clumping of cells. × 350

FIGURE 2.—Loose migratory growth. × 350



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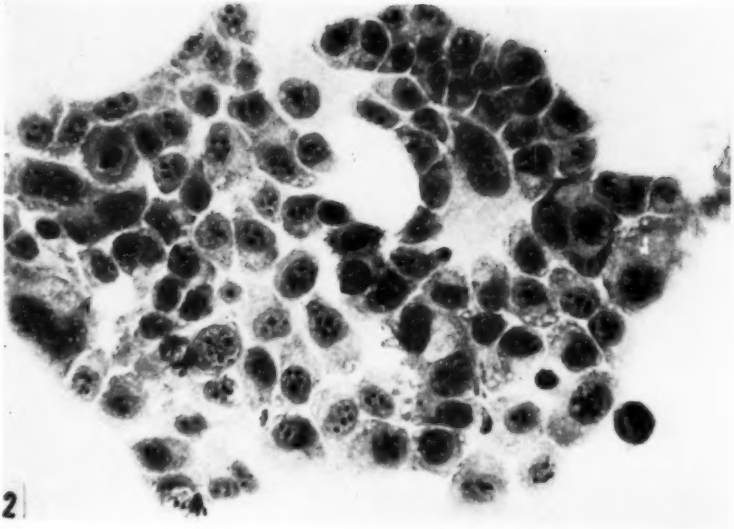

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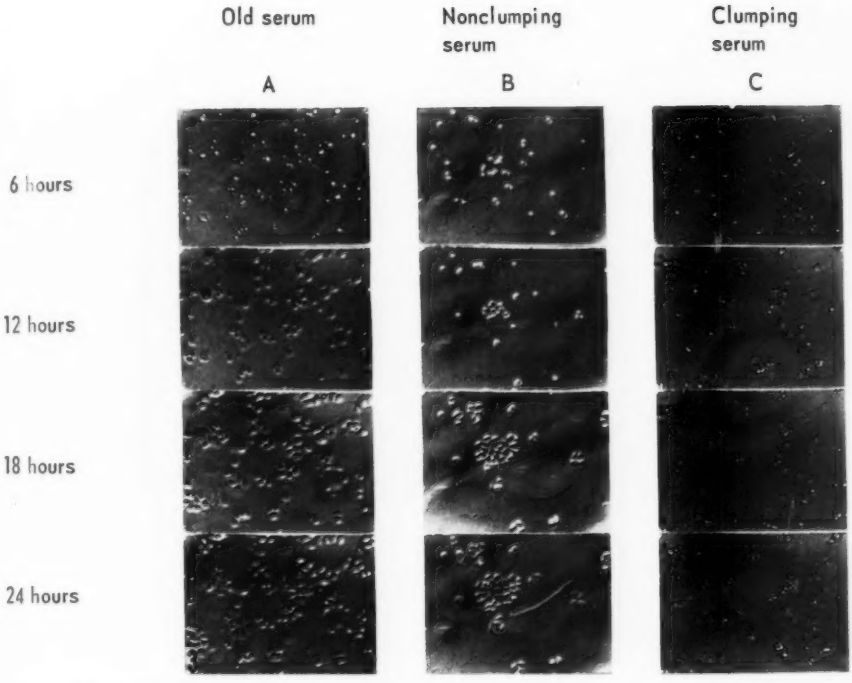
PLATE 202

FIGURE 3.—Pictures from time-lapse cinematography, taken 6, 12, 18, and 24 hours after addition of the inoculum, which illustrate differences in growth behavior of cells in different serums. × 100


Old serum
Nonclumping serum
Clumping serum
A
B
C
6 hours
12 hours
18 hours
24 hours

A Spontaneous Metastasizing Fibrosarcoma in the Golden Hamster (*Mesocricetus auratus*) With Some Observations on Tumor Recurrence and Metastasis After Transplantation [1, 2]

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SUMMARY

A subcutaneous fibrosarcoma was discovered in an untreated adult hamster. The tumor had metastasized to different organs in both thoracic and abdominal cavities. Gross and microscopic features of the tumor are described. A tumor incidence of 89 percent was obtained after subcutaneous transplantation into other hamsters. Macroscopic metastases were seen in 66 percent of the hosts during an average observation period of 96 days. Metastases occurred more frequently among hamsters bearing the larger subcutaneous tumors than among the animals with the smaller ones. The lungs and kidneys appeared to be most susceptible, while occasional metastases were observed in the lymph nodes, liver, spleen, heart, and body wall. Excision of subcutaneous sarcomas from another group of hamsters was followed by recurrences in 55 percent of these animals. The tendency to recur did not appear to be related to size of primary tumor excised. Although the incidence of metastasis was high for the hamsters with and without recurrences (100 and 73 percent, respectively), metastases were more widely disseminated in the former group.—J. Nat. Cancer Inst. 26: 1381–1393, 1961.

THE USE of hamsters in cancer research is relatively recent, only 15 spontaneous neoplasms having been recorded for this species by 1946 (*1*, *2*). Since then, additional spontaneous tumors, including both sarcomas and carcinomas, have been described by different investigators (*3–10*). In a 1951–52 survey, Dunham and Stewart (*11*) listed only two hamster tumors of spontaneous origin that were being propagated. While there have doubtless been additions to this list, the total number now under propagation is small.

Several years ago a spontaneous tumor was observed in a stock male hamster. The primary tumor, diagnosed subsequently as a fibrosarcoma, was observed at autopsy to have metastasized widely in the tumor-bearing host. Further experimentation with this tumor demonstrated that the neoplasm was highly transplantable and metastasized to a number of

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sites, including the lungs and kidneys. A description of the spontaneous sarcoma and data on its behavior after transplantation are given in the present communication. The influence of tumor recurrence on metastasis also was investigated, since the transplanted sarcoma recurred frequently after excision.

MATERIALS AND METHODS

The hamster in which the primary tumor arose was obtained from a noninbred colony. Grafts from this tumor were subsequently transplanted to other hamsters derived from the same colony. All hamsters were kept in solid-bottom cages, in air-conditioned quarters, and were maintained on Purina laboratory chow pellets and tap water *ad libitum*.

Tumors selected for transplantation were excised aseptically and grafts 1 mm in diameter prepared from peripheral areas. The grafts appeared viable grossly and were implanted subcutaneously in the nape region, one graft per hamster. A graft reaching a diameter of 2 to 3 mm, as determined by palpation, was considered a "take." Latent period was defined as the time required for the graft to reach this size.

Animals were killed after an observation period of 2 to 4 months, a portion of each grafted tumor excised, and the hamsters examined for grossly visible metastases. Tumor diagnoses were confirmed after microscopic examination.

For the study on tumor recurrences, the transplanted tumors were excised after 28 days. To minimize the possibility of a fragment "escaping" at time of excision, all sarcomas were removed intact, together with the immediately adjacent subcutaneous tissues. In no instance was evidence noted grossly of tumor invasion into the body wall. Because Tyzzer (*12*) found that intermittent pressure was a factor in metastasis, the subcutaneous sarcomas were subjected to a minimum of manipulation. The three major axes of each tumor were measured to determine tumor size.

RESULTS

Description of Primary Tumor

The tumor arose subcutaneously in the nape of the neck region in a young adult hamster obtained from a noninbred colony. The hamster was isolated and observed for several weeks, during which time the tumor continued to enlarge progressively. The animal was killed when the tumor reached a diameter of approximately 4 cm, had not yet invaded the body wall, and could be moved readily along with the overlying skin and subcutaneous tissues.

When the primary tumor was excised, the subcutaneous tissues adjacent to the tumor appeared grossly edematous and mucinous; the region was highly vascularized and contained many thin-walled blood vessels. Areas

of hemorrhage were evident throughout the mass of the tumor. Incision of the tumor disclosed an area of necrosis and hemorrhage within the center, but the outer areas appeared firm and viable.

The following microscopic observations were recorded: The tumor consisted of an abundance of closely grouped cells with a minimal amount of fibrous stroma (fig. 1). There were numerous capillaries and narrow blood sinuses. Although, in places, the cells of the tumor were fusiform and arranged compactly in a wavy pattern, the over-all picture showed a lack of organization, with cells of varying size and shape, including fusiform, angular, and rounded, distributed at random. Multinucleated giant cells were also present. Frequently, a number of mitotic figures were noted per high-power field. The nuclei stained deeply with hematoxylin and the nuclear membranes contrasted sharply with the surrounding cytoplasm. There was a wide range in size, shape, and number of hyperchromatic granules among the nuclei, each nucleus containing one or more discrete nucleoli. The nucleus : cytoplasm ratio also varied, and the cytoplasm, which contained a variable number of granules, stained slightly acidophilic to slightly basophilic. The tumor, which may have been derived from fibroblastic elements, was diagnosed as a fibrosarcoma.

At autopsy, the lungs were filled with multiple 1 to 4 mm nodules, many protruding from the surface. Histologically these nodules were metastases from the primary tumor (fig. 2). Similar nodules were located on the inner thoracic wall and diaphragm. Although there were enlarged lymph nodes in the axillary and inguinal regions, microscopic examination revealed no evidence of tumor. On the other hand, metastases could be seen grossly and microscopically in a number of organs including the liver, spleen, and kidney (fig. 3).

Subcutaneous Transplantation of Primary Tumor

The tumor was transplanted once every 2 to 3 months through 9 generations into a total of 81 hamsters (table 1). Tumor "takes" were observed in 72 (89%) of these hosts during an average latent period of 29 days. The tumors continued to grow and frequently enlarged to a diameter of 4 cm or more in 2 to 3 months without apparent invasion of the underlying musculature of the body wall or ulceration through the overlying skin. In some hamsters, these sarcomas reached a diameter of 6 to 8 cm. The 81 hamsters were on experiment an average of 96 days compared to an average of 90 days for the tumor bearers alone.

At autopsy metastases were observed grossly among many of the hamsters bearing subcutaneous tumor grafts: *e.g.*, in the lungs of 46 out of a total of 70 tumor bearers, an incidence of 66 percent (table 1). Two of the original 72 hamsters with subcutaneous tumors were not included in this calculation because the viscera in both thoracic and abdominal cavities already had been eaten when the animals were found in their cages. The average latent period for tumor "takes" was 28 days for the hamsters with metastases compared to 29 days for the 24 tumor bearers

TABLE 1.—Incidence of tumors and metastases after subcutaneous transplantation of a hamster fibrosarcoma

Total hamsters	Tumor incidence		Latent period (Average days)	Metastasis* incidence		Observation period† (Average days)	Number of hamsters with metastases in:						
(No.)	(No.)	(%)		(No.)	(%)		Lung	Kidney	Lymph-node	Liver	Spleen	Body wall	Heart
81	72	(89)	29	46	66	96	46	11	3	3	2	2	1

*Percent metastasis based on 70 tumor bearers.
†Includes all hamsters.

with none. No attempt was made to determine the earliest time of appearance of gross metastases. However, one hamster killed at 53 days already had multiple lung metastases.

The hamsters with no metastases were observed an average of 76 days. These were compared with a group of 20 hamsters with metastases and with a comparable observation period (average 77 days) to determine whether size of subcutaneous tumor at time of autopsy was related to the occurrence of visible metastases. Tumor diameters were measured along the three major axes after excision and separation of each sarcoma from the surrounding tissues. Based on these determinations, an average tumor size of $5.4 \times 3.6 \times 2.8$ cm was obtained for the hamsters with metastases, compared to $4.1 \times 3.2 \times 2.3$ cm for those with none. Thirty-eight percent of the hamsters, with no metastases and observed for 59 to 113 days, bore subcutaneous tumors with smaller diameters than the smallest tumor among the hamsters having metastases. The latter tumor measured $3.5 \times 3.0 \times 1.7$ cm after an observation period of 66 days.

Metastases were seen in different sites including the lungs, kidney, lymph nodes (fig. 4), liver, spleen, heart, and body wall (table 1). The lungs were involved in every instance of metastasis and with few exceptions bore multiple nodules. The next most susceptible site appeared to be the kidney where there was a 24 percent incidence of metastasis. Occasional metastases were found in the other sites mentioned. Since serial sections were not prepared from the organs removed at autopsy, it is possible that some metastases, especially the smaller ones, may have been overlooked.

Tumor Recurrence and Metastasis After Subcutaneous Transplantation

The sarcoma was transplanted into a total of 60 hamsters and "took" in 58 (97%) of the hosts. These tumors were carefully excised after an observation period of 28 days. Many were small, 8 mm or less in their largest diameters. These tumors and the larger ones were removed with no difficulty and each appeared to have been entirely excised. Despite this, another fibrosarcoma appeared at the site of excision in 32 (55%) of the hamsters. The recurrences were excised 28 days after removal of the "primaries" and the animals maintained an additional 9 weeks, as were the hamsters with no recurrences. Thus the total observation period was 17 weeks.

Tumor diameters and metastasis were compared in those animals which developed recurrences with those which remained free (table 2, groups R and NR). After excision of recurrences, fibrosarcomas again appeared at the site of excision in 4 of the 32 hamsters. This left a total of 28 hamsters with only one recurrence each during the entire experiment. Only the latter animals were included in group R. Two additional animals were lost through cannibalism so that the effective total was lowered to 26.

TABLE 2.—Relationship of primary tumor size to tumor recurrence and metastasis; all tumors were excised after an observation period of 28 days

Group*	Effective total, hamsters† (No.)	Primary tumor size, average diameter‡ (mm)	Metastasis incidence		Number of hamsters with metastases in:					
			(No.)	(%)	Lungs	Liver	Kidney	Rectum	Body wall	Thoracic cavity
R	26	7 × 6 × 5	26	100	26	4	4	1	—	9
NR	26	8 × 6 × 5	19	73	19	—	—	—	1	4

*Abbreviations: R = recurrence; NR = no recurrence.
†Includes hamsters with only one recurrence each during entire experiment.
‡Diameters of three major axes obtained after excision of tumors.

The "primary" tumors enlarged at different rates in groups R and NR and at autopsy varied from a low of 3 × 3 × 3 mm to a high of 15 × 12 × 8 mm. Average tumor diameters were calculated for both these groups to ascertain whether a relationship existed between primary tumor size at time of excision and the tendency to recur. These average diameters measured 7 × 6 × 5 mm and 8 × 6 × 5 mm for groups R and NR, respectively (table 2). Compared to the primary tumors, average tumor diameters measured 8 × 6 × 4 mm for the recurrences in group R after the same observation period.

Many primary tumors in group NR were relatively small at time of excision. Fourteen measured 8 mm or less (10 of these were 5 mm or less) in their largest diameter. Despite this small size, metastases arose among 50 percent of these hamsters.

Among the hamsters in group NR (table 2), 73 percent bore gross metastases at autopsy. On the other hand, the incidence was 100 percent in group R. A more widespread dissemination of grossly visible tumors was obtained for group R as compared to group NR (table 2).

DISCUSSION AND CONCLUSIONS

The data in table 1 demonstrate a high degree of transplantability for this sarcoma. In addition, this tumor metastasizes frequently after subcutaneous transplantation. The observation that average tumor diameters were smaller in those hamsters with no metastases as compared to those with metastases after a comparable observation period, and the fact that almost 40 percent of the animals with no metastases bore subcutaneous tumors smaller in diameter than the smallest tumor in the hamsters with metastases suggest a direct relationship between tumor size and the occurrence of metastases. These findings agree with those of other investigators (*13–17*) who observed a similar relationship. It is apparent, however, that size alone is not the only factor in the development of metastasis, for some hamsters with no visible metastases in the present experiment bore subcutaneous tumors as large as 4 to 6 cm in diameter. Duration of tumor residence in a host has also been related to metastasis development (*12–14, 17–21*). While no attempt was made to investigate this relationship in the present study, probably still other factors are concerned, for there were no visible metastases in a number of hamsters bearing large subcutaneous tumors despite observation periods ranging from 106 to 146 days.

Tumor nodules were seen grossly in a number of organs including the lungs and kidneys (table 1). Significantly more instances of metastases were noted in the lungs than in any other organ. A similar result was obtained by other investigators after transplantation of sarcomas into hamsters (*6, 22, 23*), mice (*24*), and rats (*25*). In some studies with transplantable sarcomas in the hamster, however, metastasis occurred more frequently in the lymph nodes than in the lungs (*18, 22, 26*). Dunn

(*27*) reported that metastasis in the kidneys occurred rarely in the mouse except in leukemia. Although sarcomas have been transplanted repeatedly in the hamster, only an occasional animal had metastases in the kidneys (*1*, *2*, *6*, *18*, *22*). Crabb (*18*) implanted a polymorphous-cell sarcoma into hamsters and noted only 3 out of 115 tumor bearers with metastases in the kidneys. The development of grossly visible metastases in this organ in 11 out of 46 tumor bearers (table 1) was unanticipated. There was a similarly high incidence of metastasis in the kidneys in a related investigation[4] in which the same sarcoma was transplanted intramuscularly. In the latter experiment, 7 of 22 tumor bearers (32%) bore grossly visible kidney metastases during an average observation period of 90 days. It may be that this sarcoma is disseminated in the form of small aggregates and thus favors passage through the capillary beds of the lungs. In support of this possibility, multiple 1 to 4 mm tumor nodules frequently protruded from the periphery of the lungs. Knisely *et al.* (*28*) injected the VX2 rabbit carcinoma intravenously in the form of small tumor particles and obtained frequent tumors in the liver and kidneys. Sugarbaker (*29*) implanted cells from different tumors into the left ventricle of rats and observed tumors in the kidneys and adrenals of many animals. Thus the kidneys appeared to be susceptible to metastasis, once the tumor cells had entered the arterial circulation. While this may be sufficient to explain the kidney metastases in the present experiment, it is also possible that the hamster kidney is especially susceptible to the particular sarcoma under investigation.

Subcutaneous tumors recurred readily after their excision (table 2). Although the reason for this is not known, it may well be that these recurrences were the result of incomplete excision or "seeding" of the wound.

Based on average tumor diameters, primary tumor size in groups R and NR at 28 days was essentially the same (table 2). Thus, whether tumors recurred did not appear to be related to size of the primary tumors at time of excision.

No attempt was made to determine the period during which metastasis occurred. Thus, it is not known whether the sarcoma cells were disseminated at the time of excision of the primary tumors or before. However, it is apparent that in some instances, at least, this occurred when the subcutaneous neoplasms were small and within a relatively brief observation period. In the experiments of Ketcham *et al.* (*21*), metastases were similarly observed among mice when the tumors they were bearing were removed after 21 days. In the latter study, however, the tumors measured 15 to 20 mm at this time and thus were considerably larger than the 3 to 15 mm tumors excised in the present investigation.

The more widespread distribution of metastases in group R as compared to group NR (table 2) suggests an enhancement in metastasis. It may well be that this resulted because animals in group R bore tumor tissue subcutaneously for an over-all longer period than did animals in group NR. It is evident that under these circumstances a greater opportunity would

[4] M. Klein—unpublished data

have been provided for dispersal of tumor cells. Also, it will be recalled that subcutaneous tumors were excised once in group NR but twice in group R (removal of recurrences as well as primary tumors). It is possible that dissemination of tumor cells in the latter group was increased as a result of this added excision.

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All sections illustrated in the following plates were stained with hematoxylin and eosin after fixation in Tellyesniczky's fluid. × 100

PLATE 203

FIGURE 1.—Section from primary hamster fibrosarcoma. *Note* densely grouped tumor cells and minimal stroma.

FIGURE 2.—Two fibrosarcoma nodules observed in lungs of primary tumor bearer.

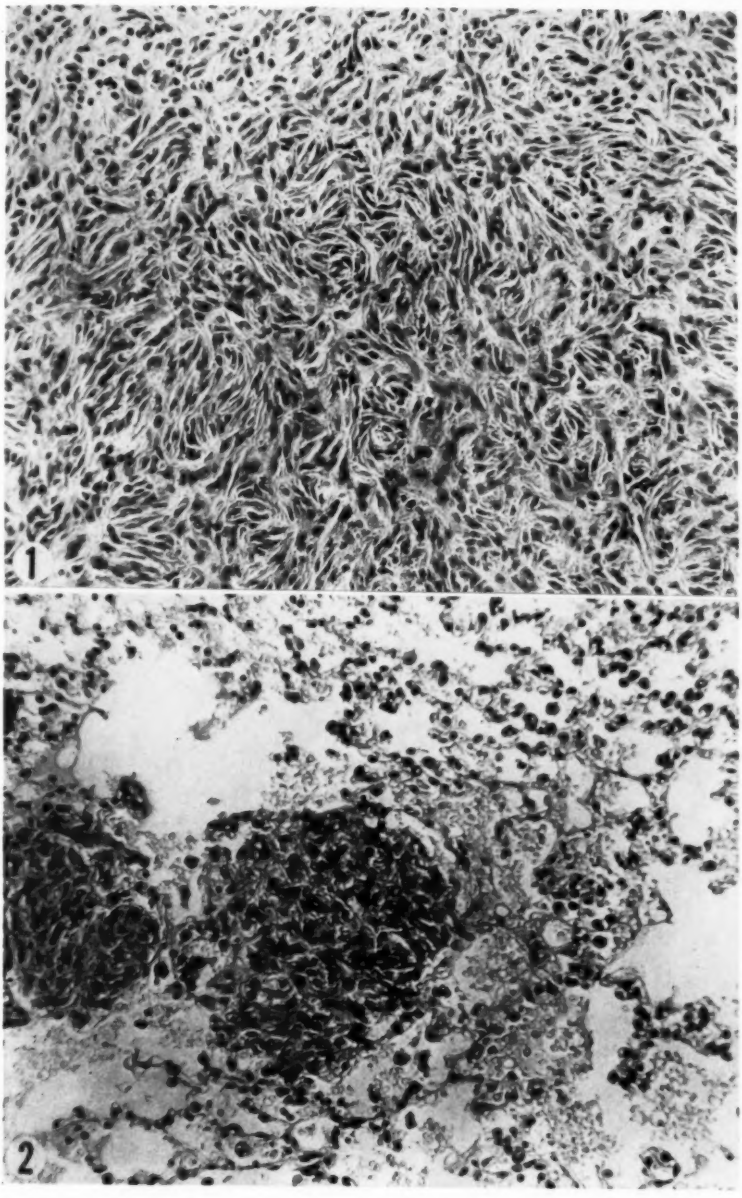

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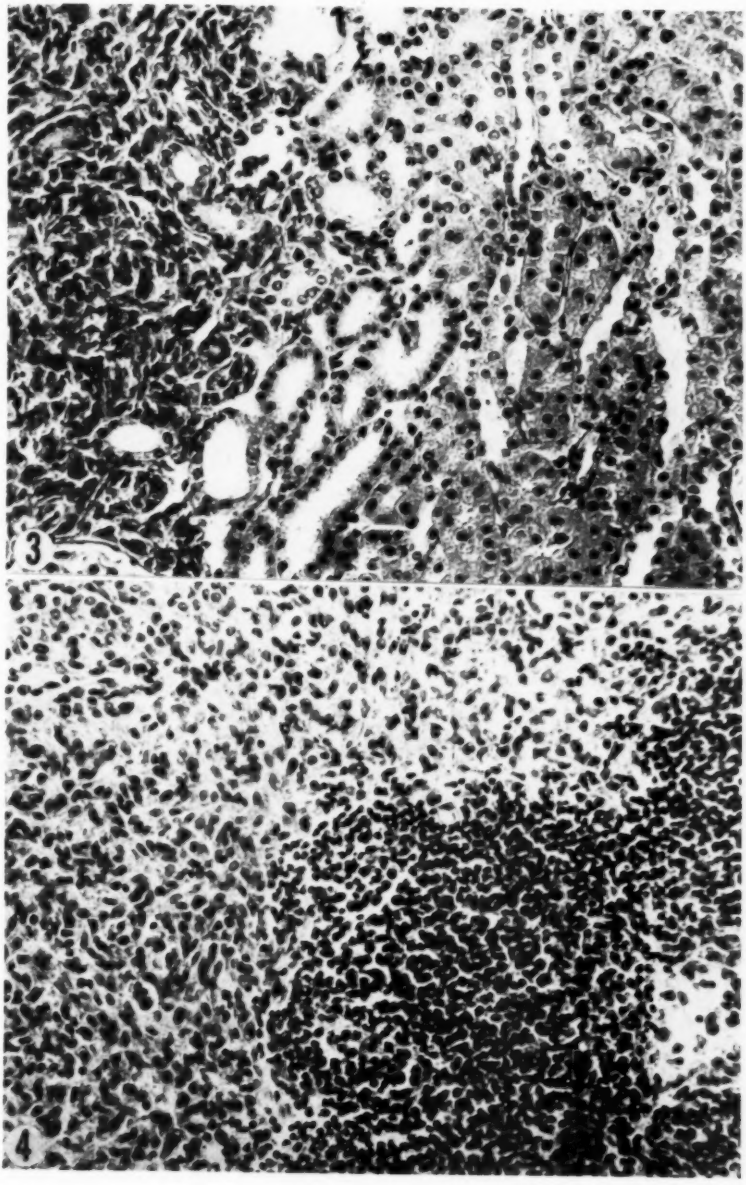
PLATE 204

FIGURE 3.—Metastasis of primary fibrosarcoma in kidney.

FIGURE 4.—Metastasis of transplanted fibrosarcoma in lymph node. *Note* numerous mitotic figures scattered throughout tumor mass.


3
4

Histology of Sex-Specific Graft Rejection [1, 2]

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SUMMARY

Female mice carrying isologous male skin grafts were serially killed for study of the histology of sex-specific rejection. A reaction had already started 8 days after grafting, much earlier than gross inspection suggests, and only a few days later than the reaction to homografts. Specifically sensitized females may show evidence of a sex-specific reaction as early as 4 days after grafting.—J. Nat. Cancer Inst. 26: 1395–1403, 1961.

EXPERIMENTS CONCERNED with sex-specific rejection—the rejection of male skin grafts by isologous female mice—are usually of long duration. The onset of this rejection has been reported between the 29th and 65th days (*1*), between the 31st and 55th days (*2*), between the 15th and 65th days (*3*), and as late as the 109th day in mice of the A strain (*3*). The course of these experiments is even more protracted when second-set phenomena are under study. Another handicap in these experiments is the lack of a sharp endpoint. Scaliness, baldness, or erosion of the graft surface usually has been taken as endpoints; the events prior to the occurrence of these signs resembled on gross observation those seen in autografts. The experiments reported in this communication were undertaken to define an endpoint on a histologic level that would be more precise and presumably yield earlier rejection data.

MATERIALS AND METHODS

Animals.—Donors and recipients of sex-specific grafts (skin of male donors transplanted to isologous females) were F_1 hybrids of the C57BL/6 strain, the other parent usually of the BALB/c strain. Donors and

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[3] With the technical assistance of E. Goff.

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recipients of homograft controls were of the same sex. The donors were F_1 hybrids of the C57BL/6 strain, the other parent of either the ST or the CBA strain; BALB/c mice were used as recipients. C57BL/6 F_1 hybrids were used as isograft controls.

Method of grafting.—The technique of grafting has been previously described (4) and involves placing a portion of tail skin in "open style" onto the trunk, the transverse grooves of the graft running in craniocaudal direction of the trunk, the tail hair thereby being tilted at right angle to the hair of the trunk. The grafts measured approximately 5 × 7 mm, and on average weighed 12.6 mg. The graft was placed onto the intact panniculus carnosus of the recipient's trunk, which is covered by a thin, shaggy, vascularized layer of loose, areolar tissue occasionally harboring an isolated root of a hair follicle. This results in a fair fit in height, since the level of the upper graft surface approximates, or slightly protrudes, that of the upper surface of the adjacent host skin.

Tail skin was used as a graft source because of its distinct surface and pigmentation characteristics. The peculiar spacing of hair follicles at this site, the dorsal pigmented streak (in nonalbino mice), and the coarsely grooved pattern of the dermal collagen render it particularly suitable for gross and microscopic study. As the hair follicles traverse the dermis they arrange themselves in loose triads, a distinctive characteristic on transverse sectioning. On longitudinal sectioning the surface has a shingle-like arrangement: A gently ascending slope in craniocaudal direction is followed by a short, steep drop, forming either a convex or concave surface which the hair shafts pierce in a very acute angle to emerge between two rows of shingles. Tail skin lacks a panniculus carnosus and subcutaneous tissue. Instead, the dermis consists of two fairly distinct layers. The upper is composed of closely packed, intertwining bundles of collagen arranged in an even plane at the interface with the lower dermis; it appears barely vascularized except in the loose reticular tissue surrounding the upper portions of the hair follicles. The arrangement of the lower dermis is less compact and less regular. It contains follicular roots, nerves, a small amount of fatty tissue, capillaries, and one or more medium-sized to large vessels coursing in anteroposterior direction—the usual site of intravenous injections—and of special interest in the study of vascular phenomena, such as the "white graft" reaction.

Method of observation.—Grafts were not biopsied. Bandages were not removed until the time of killing, to minimize any secondary changes due to trauma. At this time the entire graft, an adjacent generous square rim of host skin separated from the graft by an open moat, and the underlying panniculus were removed, placed on a piece of paper towel, and fixed in 10 percent formalin. The fixed specimen was blocked parallel to the tail grooves, in most instances, and embedded on edge. Most sections were stained with hematoxylin and eosin, with an occasional elastic tissue (Weigert) and Prussian blue stain.

Sensitization procedures.—Mice were sensitized either by skin grafts or injections of splenic cells. The sensitizing grafts were placed on the

left and the challenging grafts on the right side of the trunk. We obtained spleen cells, usually in a dose of 20×10^6 cells, by "milking" the organ after hemisectioning, suspending the "milk" in Ringer's solution, and injecting it intraperitoneally through a 27-gauge needle.

OBSERVATIONS

Isograft Controls

Between the 1st and 30th days, 38 isograft controls were killed serially, most of them during the 1st week of grafting. The observations were similar to those reported by other investigators in other species (*5*, *6*). A blood supply was established early, and anastomoses between graft and host vessels played a part. There was no incontrovertible evidence for the ingrowth of host capillaries; there were neither "perpendicular sprouts" (*5*) as described for human autografts and homografts, nor was there any degree of angioblastic proliferation at the host-graft interface. This finding is in line with the observation of Edgerton that "a much more significant role seems to be played by the actual vascular framework of the graft itself." (*7*). Anastomoses between host and graft vessels, though demonstrable, also were not seen in profusion. It seems that a few host-graft anastomoses suffice for an adequate blood supply, presumably by virtue of ample anastomoses between graft vessels. Thrombosis of graft vessels was not uncommon at the end of the healing period, and probably due to lack of use.

Homograft Controls

Between the 3d and 15th days, 125 homograft controls were killed. The observations were similar to those reported by Medawar in rabbits (*6*). Cellular infiltration in excess of that in isografts was seen as early as the 5th day. It preceded vascular phenomena (dilatation of graft vessels and hemorrhage), which preceded epithelial destruction. Evaluation of the viability of the graft epithelium was at times difficult. There were variations between different sections of the same graft and even between different fields within the same section. Not infrequently, the epidermal appendages were well preserved, while the surface epithelium was in a state of advanced degeneration. During the first half of the 2d week overgrowth of host epithelium also complicated interpretation.

In specifically sensitized hosts, rejection occurred either as a "white graft" phenomenon (*8*) or as an accelerated rejection. When it was not of the "white graft" variety, the large vessels of the lower dermis were dilated and often thrombosed on the 6th day, the capillaries were intensely engorged, and recent interstitial hemorrhage was widespread. In the "white grafts," the large vessels were collapsed, and at times only recognizable with elastic tissue stains, which thereby provided a sharp endpoint

for this phenomenon. The grafts appeared completely avascular, though an occasional capillary cluster was seen in the lower dermis, and conceivably represented invading host vessels to which anastomosis with graft vessels was denied.

Sex-Specific Grafts

Between the 1st and 30th days, 133 mice with sex-specific grafts were killed. A host reaction was evident *8 days* after grafting. Lymphocytes and fibroblasts accumulated around hair follicles (fig. 1) traversing the upper dermis to form conspicuous collars that stood out against the surrounding dermal tissue. Lymphocytes and fibroblasts were also in the lower dermis but more diffusely distributed. An occasional vessel was filled with lymphocytes. The epidermis and sebaceous glands were undistinguishable from those of isografts.

On the *10th day* perifollicular collars were more conspicuous. The basal layers of the follicles and also the epidermis were edematous and showed early migration of inflammatory cells. Infiltration of the lower dermis was more intense, and a few clusters of large mononuclear cells, resembling plasma cells, were in the pannicular tissue of the host and the overlying graft dermis (fig. 6).

The perifollicular infiltration had reached its peak by the *12th day* (fig. 2), with profuse infiltration of the entire follicle and advanced degeneration of the epithelium. Occasional granulomas had formed around degenerating follicles. Infiltration of the lower dermis was intense, neutrophils being more conspicuous than on previous days. Most sebaceous glands had disappeared by this time, though there was an occasional residual cluster at later stages. The epidermis was still hyperplastic, edematous, and infiltrated by inflammatory cells (fig. 5).

The cellular infiltrate began its decline around the *14th day*. Some hair follicles were replaced by nests of atrophic epithelial cells, fibroblasts, lymphocytes, and dilated capillaries. Inflammatory cells also began to disappear from the upper dermis. The appearance of the epidermis varied. Foci of edema, inflammation, and atrophy alternated with hyperplastic areas, the latter probably being early evidence of regenerative activity of host epidermal cells.

Progression of graft rejection thereafter varied in speed from graft to graft, rendering a day-by-day description difficult. The end results also differed. Slow replacement of the connective-tissue matrix of the graft resulted in a smooth scar approximately the size of the graft, while more rapid replacement produced a contracted, pitted scar.

Observations on the *16th, 18th, and 20th days* showed replacement of most hair follicles by granulation tissue. A few stragglers still showed epithelial-cell nests infiltrated with lymphocytes and neutrophils. The epidermis was uneven; in places it appeared well preserved, of normal thickness, though diffusely infiltrated by inflammatory cells; elsewhere it was atrophic; again, in other places there was replacement by hyperplastic

host epithelium, frequently intermingled with inflamed, degenerating graft remnants. In general, regeneration was slow. Pegs of host epithelium sometimes dipped into pits vacated by degenerated graft follicles, and areas of cornification were seen within these pegs (fig. 3). This raised the possibility of the production of new follicular primordia by the host, any resultant hair being slanted in the direction of that of the rejected graft. If such replacement were to occur, one might wonder about the reliability and the *direction* of hair growth as a criterion of graft survival. The cellularity of the lower dermis and graft bed had decreased only little by this time, and projections of granulation tissue extended upward to encompass any follicular remnants (fig. 4).

Observations from the *22d to 30th days* indicated that the process of final rejection and replacement was slow when compared with the rapid onset of rejection early during the 2d week. There was much variation from graft to graft. Evidence of residual inflammation in the lower dermis and graft bed was common at the 30th day, but hair follicles usually had disappeared by that time.

In summary, the fate of sex-specific grafts was essentially divided into four 1-week phases: no changes during the 1st week; a massive, rather sudden inflammatory response during the 2d week; replacement of most components during the 3d week, with decrease of the inflammatory response, and regenerative activity of the host; and a tidying-up period during the 4th week, which extended into the 2d month.

Sex-Specific Grafts in Sensitized Hosts

Previous sensitization of a female mouse by isologous male tissue resulted in an accelerated host response. Evidence of this response was seen as early as the *4th day*. The graft bed showed early infiltration by large and small mononuclear cells and occasional neutrophils, and there was also some infiltration of the lower dermis by similar cells. This was more intense on the *5th day*, and by the *6th day* the infiltrate had reached the upper dermis, and small perifollicular collars were frequently, but not always, observed. As in first-set *homo*grafts, the epithelial structures of the graft appeared as yet unaffected; there was regenerative hyperplasia of the surface and follicular epithelium, and many sebaceous glands had recovered.

A distinct change was noted on the *8th day* if the interval between sensitization and challenge was as long as 30 days. Regenerative activity of the epithelial structures had ceased. The basal layer of epidermal and follicular epithelium showed edema and round-cell infiltration, and foci of necrosis and atrophy were seen. Sebaceous glands had disappeared. The dermis, particularly in its lower layer, was intensely infiltrated with lymphocytes and fibroblasts, and blood vessels were frequently dilated and thrombosed. A sharp line of demarcation had been formed against the edematous and congested host panniculus.

By the *10th day*, the epithelial structures of the graft were almost

completely destroyed, but replacement by host epidermis had not yet occurred. Inflammatory cells had virtually disappeared from the upper dermis which had begun to thicken and to sclerose. There was still ample inflammatory activity in the lower dermis which, as on the *8th day*, was distinctly delineated from the host panniculus.

Over- and ingrowth of host epidermis was apparent by the *12th day*, while the appearance of the graft dermis and host panniculus was essentially unchanged. By the *14th day*, the edema of the host bed had disappeared; the interface between graft and host had become less distinct, with approximation of the lower graft dermis to the pannicular muscle.

If the interval between sensitization and challenge were merely 8 days, graft rejection, though accelerated, was less precipitous. On the *8th day*, the epithelium of the test graft was still well preserved, hyperplastic in places, and showed only focal inflammatory changes. Perifollicular collars were still present, and the picture resembled that of first-set, sex-specific grafts. Neither changes in dose of sensitizing cells, in interval between sensitization (or first graft) and test graft, nor hypersensitization resulted in the "white graft" phenomenon.

DISCUSSION

Our observations indicate that sex-specific rejection of skin grafts starts much earlier than gross inspection suggests. A massive inflammatory response is under way early during the 2d week after grafting, and active rejection is virtually terminated at the end of that week. The onset of the rejection against sex-specific grafts lags behind that against homografts for only 2 to 3 days, though the reaction proceeds at a slower pace. Scaliness, baldness, or erosion—the endpoints suggested by gross inspection—represents merely a healing process. In sensitized animals evidence of rejection may be noted by the *4th day*, earlier than in first-set homografts. Sex-specific white grafts were not observed.

Microscopic evidence of a sex-specific host reaction merely indicates the existence of a host response. This response need not necessarily be successful since it is conceivable that some grafts may be able to withstand the onslaught.

Early rejection is not restricted to grafts of tail skin, but is also seen with trunk skin. However, the perifollicular infiltration characteristic of sex-specific rejection stands out more distinctly in tail skin grafts.

Since most of the histologic events that bring about sex-specific rejection escape the naked eye, one may wonder whether females of strains in which rejection is reported to occur only in some animals, and relatively late, do not reject universally. Rejection would occur slowly, with creeping replacement of the structures responsible for surface characteristics, and escape gross observations altogether. If this were true, only these animals woud be considered rejectors in which the process was relatively speedy. This possibility could be ruled out by retransfer of male A skin from a

first "nonrejecting" female A recipient to an (A × C57BL)F_1 female which would reject this graft. We have approached this problem from a histologic point of view. Female strain A mice carrying male A grafts were serially killed, as late as the *18th day*, in order to detect evidence of universal rejection (unpublished observation). We have not found such evidence, and in consequence had to adhere to the assumption that there are indeed reacting, nonreacting, and partially reacting strains. Likewise, female (BALB/c × C57BL)F_1 mice, rendered tolerant of male isologous skin by irradiation (500 r) and intraperitoneal injection of 42×10^6 male splenic cells and subsequently grafted with isologous male skin, failed to reveal histologic evidence of graft rejection.

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FIGURE 1.—Hair follicles in upper dermis of sex-specific graft after 8 days, showing early perifollicular round-cell infiltration. (Donors and recipients of illustrated grafts are (C57BL/6 × BALB/c)F_1 hybrids or their reciprocals.) Hematoxylin and eosin. × 100

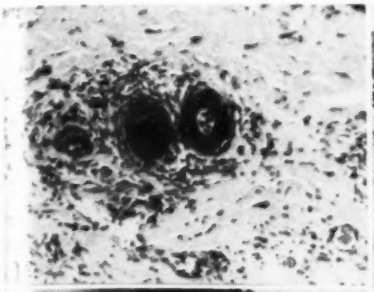
FIGURE 2.—Perifollicular collars of sex-specific grafts after 12 days, showing early obliteration of follicles. *Note* absence of involvement of remainder of upper dermis. Hematoxylin and eosin. × 100

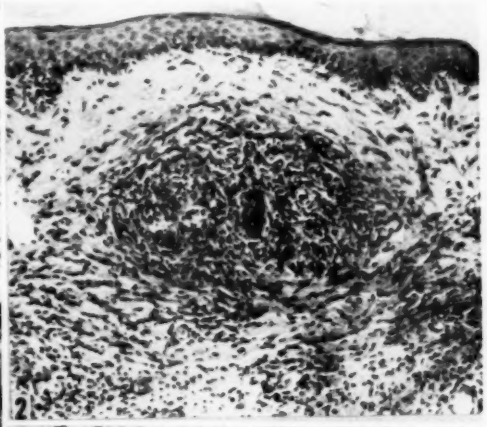
FIGURE 3.—Regenerating host epithelium filling pits vacated by degenerated follicles of 24-day-old, sex-specific graft. Hematoxylin and eosin. × 180

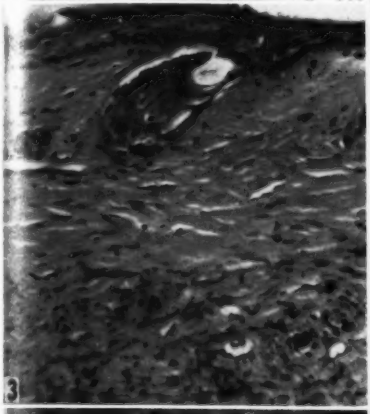
FIGURE 4.—Residual of 20-day-old, sex-specific graft, showing dentate effect caused by replacement of graft follicles by granulation tissue. Hematoxylin and eosin. × 100

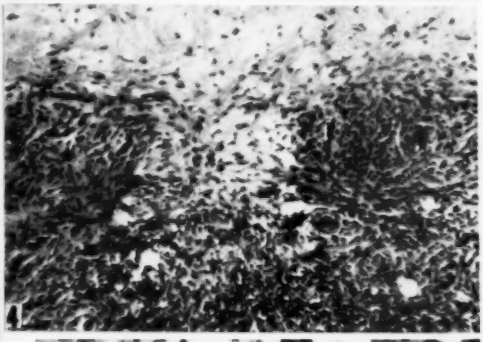
FIGURE 5.—Edge of hair follicle on 12th day of sex-specific graft, showing infiltration with inflammatory cells. *Note* cluster of well-preserved cells of sebaceous glands in center of follicle. Hematoxylin and eosin. × 650

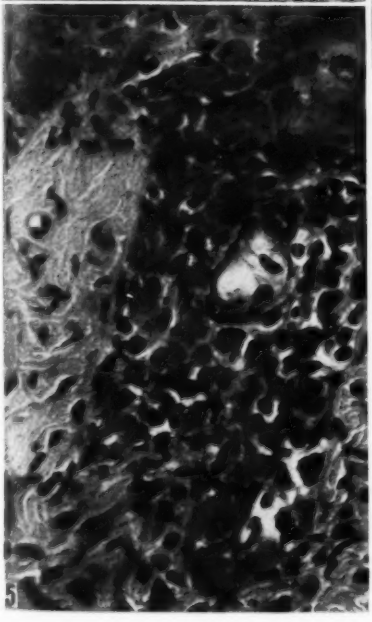
FIGURE 6.—Mononuclear infiltrate in suprapannicular tissue of host 10 days after transfer of sex-specific graft. Hematoxylin and eosin. × 750











Clone Strains of Pig Kidney Cells With Drug Resistance and Chromosomal Markers [1, 2]

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SUMMARY

Two strains of pig kidney cells have been isolated that are resistant to aminopterin and 2,6-diaminopurine, respectively, and that can be identified cytologically by characteristic marker chromosomes. Drug resistance and chromosome patterns in these lines were stable during short-term serial subculture in normal media. Stabilization of marker strains by routine subcloning is recommended, with the use of frozen type specimens for reference or recovery as needed.—J. Nat. Cancer Inst. 26: 1405–1411, 1961.

THE POTENTIAL usefulness of cell cultures for genetic studies has long been recognized. As model systems for the analysis of somatic variation, they offer the advantages of selected material and the ready manipulation of population parameters. Through recent advances in the techniques of cell culture, established cell strains (*1–5*), defined media (*6–8*), cloning procedures (*9*, *10*), and quantitative methods for growth measurement (*11–13*) are now available as aids in experimental design. Although the application of these systems to the study of cellular heredity has just begun, a number of specific characteristics have been described that might form the basis for systematic genetic studies *in vitro*. These include altered morphology (*14*), glycolytic properties (*15*), nutritional requirements (*16*), drug resistance (*17–19*), and ability to produce tumors in susceptible hosts (*14*, *20*). Experience in microbial genetics and in other more conventional systems has shown that combinations of markers within a given strain often facilitate genetic analysis. Multiple markers can serve to identify isolated cell populations as well as to check for segregation or genetic transfer under experimental procedures. The purpose of this paper is to show how at least 2 such markers can be introduced as stable characteristics into clone lines of pig kidney cells. Previous studies (*21*) have shown that cell lines can be uniformly labeled with unique marker

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chromosomes, produced by exposure to X irradiation followed by clonal isolation of survivors. Other experiments have demonstrated that clone strains of cells resistant to antimetabolites can be obtained by appropriate selection systems (*22*), that resistance is specific (*19*), and that marker characteristics persist for extended periods in the absence of the corresponding drug (*23*). In the experiments described, drug-resistant variants have been isolated as clonal derivatives from cell strains with unique chromosome patterns, which thus combine the 2 types of markers. Since the methods that have been used are general in application, it appears feasible to extend the process of labeling to additional markers and other cell strains.

MATERIALS AND METHODS

Culture techniques.—All experiments were performed with derivatives of a clone line of pig kidney cells (PK 14). The origin of this and related strains has been given in preceding papers (*13*, *20*). Stock and experimental cultures were maintained as static monolayers and subcultured by standard procedures in prescription bottles as described previously (*13*). The medium employed consisted of 5 percent lamb serum, 50 percent medium 199 (*6*) and 45 percent Earle's balanced saline. Antimetabolites where used were incorporated in the saline fraction. Fluid changes were made 3 times per week and subcultures or terminal counts performed with the aid of a Coulter cell counter (*13*).

Derivation of marker strains.—PK 14 cells were exposed to X irradiation by one of us in the course of another study on chromosome variation in cell populations. Survivors from a population receiving 500 r (250 kvp) were continued in recovery cultures, and, after 3 further subcultures, clones were isolated by plating procedures (*10*). When chromosome preparations were subsequently examined, a number of these clone lines showed variant karyotypes with marker chromosomes (*21*). Two of the marker lines, C23 and C26, were selected for additional treatment with antimetabolites, by a method based on procedures described earlier for the isolation of drug-resistant variants (*22*). Cultures of 3.0×10^6 cells were established as monolayers in T-60 flasks, in the presence of 0.1 μg/ml aminopterin (C23) or 100 μg/ml 2,6-diaminopurine (C26). Survivors from these treatments proliferated secondarily and by 4 weeks had formed a series of resistant colonies in the culture flasks. Subcultures were made from these populations and clonal sublines were isolated by plating procedures. The clonal lines selected for further study were designated R206 (isolated from strain C23 with aminopterin), and R208 (originating from strain C26 exposed to 2,6-diaminopurine).

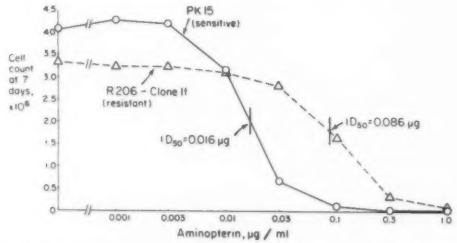
Chromosome preparations.—Cultures for chromosome studies were treated with colchicine and hypotonic saline. This treatment was followed by fixation in acetic acid, orcein staining, and preparation of cell squashes. Details of the techniques in use have been described in previous publications (*21*, *23*).

RESULTS

Drug Resistance in Marker Lines

The results of earlier investigations on drug resistance in pig kidney cells (*19, 22, 23*) form a background for interpretation of the degree of drug resistance observed in the marker strains R206 and R208. It has been shown that toxicity tests under standardized conditions consistently show a differential between sensitive and resistant populations in terms of the concentration of drug required for 50 percent growth inhibition at 7 days. This endpoint was chosen largely for convenience and similar differences can be demonstrated in younger or older cultures.

With this general procedure, the degree of resistance in strain R206 to aminopterin was compared to that of PK 15, a sensitive line of pig kidney cells that had not previously been exposed to this drug. Groups of experimental cultures from each cell line were initiated at 0.2×10^6 cells, with the use of graded concentrations of the inhibitor, and these were maintained for 7 days. Terminal cell counts were then made and average values were plotted in the form of dose-response curves (text-fig. 1). From these data and other experiments that gave substantially similar results, it is clear that the level of drug required for 50 percent growth inhibition at 7 days (ID50) is greater for R206 populations than for PK 15 cells not previously in contact with aminopterin.

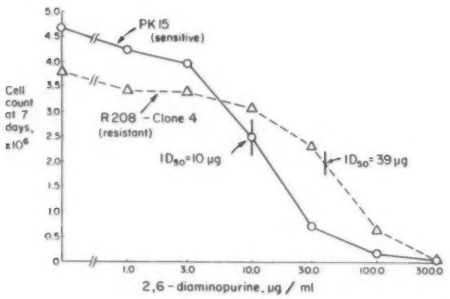


TEXT-FIGURE 1.—Dose-response assays with sensitive and resistant lines of pig kidney cells. Each point represents an average value for cell counts from 3 replicate cultures.

Similar assays were carried out with strain R208 and PK 15 cells; in these assays we used 2,6-diaminopurine instead of aminopterin. The results obtained in a typical experiment are shown in text-figure 2. The dose-response curves for the resistant and sensitive strains can be readily distinguished on the basis of ID50 values. In further experiments R208 cells showed no evidence of cross-resistance to aminopterin, nor were R206 cells refractory to 2,6-diaminopurine.

Chromosome Studies

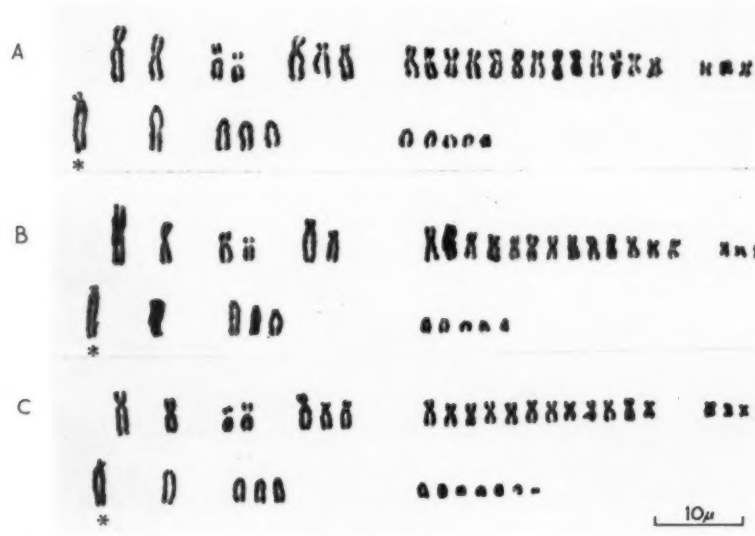
Strain R206.—Karyotypic studies were made on the parental line, C23, before exposure to aminopterin (*21*). The population consisted primarily



TEXT-FIGURE 2.—Dose-response assays with sensitive and resistant lines of pig kidney cells. Each point represents an average value for cell counts from 3 replicate cultures.

of near-diploid cells (95%) with a modal chromosome number of 37. All cells exhibited a distinctive chromosome marker in the form of a long, acrocentric chromosome not found in the original PK 14 line before X irradiation (*21*).

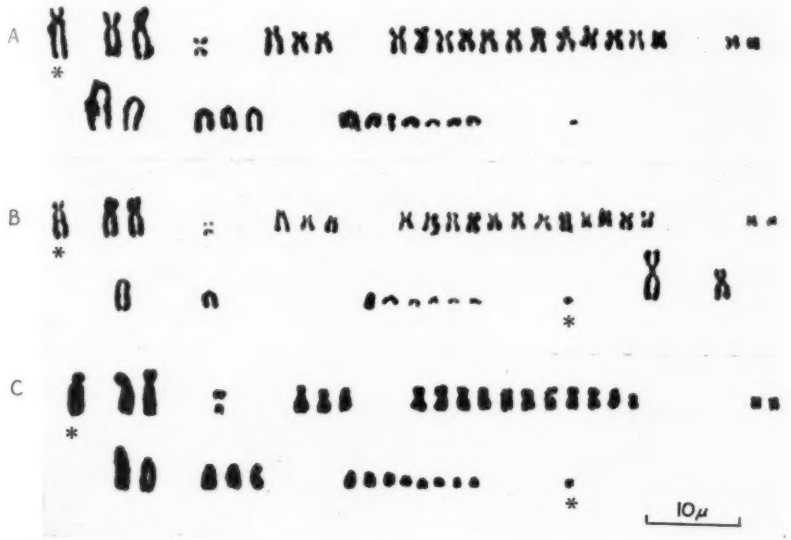
After the isolation of R206 as an aminopterin-resistant derivative of C23, chromosome preparations were again made and the karyotype analyzed by means of photo-idiograms (text-fig. 3). The characteristic chromosomal marker (long acrocentric) found in the parental C23 strain could be readily demonstrated, and persisted without change in drug-



TEXT-FIGURE 3.—Photo-idiograms of 3 representative cells from strain R206 (resistant to aminopterin). *Note* marker shown by asterisk in each, at *left side, bottom row.*

resistant cells. Since several smaller chromosomes were lost, however, there was a modal chromosome number of 34 as compared to 37 for the parental C23 line. The chromosomes in strain R206 cells were characteristically decoiled and in general the chromosome cytology was poor. An unusually high number of binucleate cells was observed (1–5%).

Strain R208.—In the strain of origin (C26) before drug treatment, all cells were characterized by 2 chromosomal markers: 1) a long acrocentric chromosome that frequently showed secondary constrictions in the long arm and 2) a minute chromosome (*21*). Text-figure 4 shows these markers in photo-idiograms of 3 drug-resistant cells from strain R208. Both characteristic chromosomes are clearly evident and have persisted without change, though in some cells the expression is weak because of poor cytology. A modal chromosome number of 33 was observed for strain R208, with a loss of several smaller chromosomes, as in strain R206.



TEXT-FIGURE 4.—Photo-idiograms of 3 representative cells from strain R208 (resistant to 2,6-diaminopurine). *Note* markers which are shown by asterisks: 1) minute chromosome at *extreme right, lower row,* 2) acrocentric chromosome with constriction, *left side, upper row.*

DISCUSSION

Both markers studied in these experiments appear to represent stable modifications in characteristics of the corresponding cell lines. The marker chromosomes of R206 and R208 were unchanged by drug treat-

ment and occurred uniformly in all cells. Similarly, drug resistance in these cell lines persisted during serial subculture in the absence of inhibitor and could be demonstrated in further clonal derivatives. Since spontaneous variations can occur over longer periods of culture, however, both in karyotype (*21*) as well as in pattern of drug resistance (*23*), special precautions are needed to insure the stability of marker strains. It appears practicable to stabilize these lines indefinitely through frequent cloning, together with the use of frozen samples for reference or recovery of the original cell types.

The chief disadvantage of the combined use of drug resistance and chromosome analysis lies in the inferior quality of chromosome preparations obtained from many of the resistant lines. This deficiency may be a reflection of the lowered chromosome number, observed here with strains R206 and R208, and previously noted in strains with poor cytology (*23*). Whether variants with improved properties in this respect can be obtained by selection from hypodiploid populations remains to be determined. The initial loss of chromosomes may be related to the acquisition of resistance, possibly by the deletion of genes responsible for incorporation of the metabolic antagonists into sensitive cells. In this way nondisjunction, which occurs frequently among cells *in vitro*, could provide a mechanism for the appearance of drug-resistant variants.

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Chromosomes of Tumor Cells. II. Effect of Various Liver Carcinogens on Mitosis of Hepatic Cells [1]

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SUMMARY

A comparative cytological study of the effects of various liver carcinogens and noncarcinogenic derivatives on mitosis was undertaken. 3′-Methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and thioacetamide (TAA) were chosen as substances of high carcinogenic activity; 4-monomethylaminoazobenzene (MAB) as a substance of moderate activity; *o*-aminoazotoluene (AAT), 4′-methyl-4-dimethylaminoazobenzene (4′-Me-DAB), ethionine, and thiourea as weak carcinogens; and 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB) and 4-aminoazobenzene (AB) as two samples of noncarcinogenic azo dyes. Their ability to provoke a proliferation of hepatic tissue, to induce the formation of highly polyploid cells, and to cause mitotic irregularities was examined. A correlation existed between the carcinogenic activity of a substance and its ability to induce a proliferation and a relatively large number of mitotic irregularities in the precancerous liver, which lead to a genetically heterogeneous cell population. 2-Me-DAB is noncarcinogenic, although it can produce mitotic irregularities in the regenerating liver, because it lacks the ability to stimulate cell proliferation. AB induces neither a chromosome damage or proliferation. Thus, these noncarcinogenic azo dyes lack the ability to produce a genetically heterogeneous cell population in the liver. The type of mitotic irregularities, varying from chromosome fragmentation to incomplete spindle function, was similar in all precancerous livers independent of the molecular structure of the carcinogens used. The chromosome injury induced by the carcinogens can persist in a latent form over several months. It was concluded that hepatomas are only produced when both factors are present: an injury to the chromosomes and an active cell proliferation.—J. Nat. Cancer Inst. 26: 1413-1427, 1961.

MITOTIC ABNORMALITIES are involved in the formation of new cell lines containing specific chromosome complements (*1–4*). A number of these tissue-culture lines have the properties of malignant cell populations (*4, 5*). These observations on tissue cultures were regarded as a model of carcinogenesis *in vivo* (*4*). The conditions found in precancerous stages

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during liver carcinogenesis revealed a similarity to this model experiment (*6*). The application of 4-dimethylaminoazobenzene (DAB) [4] results in the induction of mitotic irregularities in hepatic tissue, the formation of a heterogeneous cell population and the development of hepatomas consisting of cells with abnormal amounts of deoxyribonucleic acid (DNA) (*6*), aneuploid chromosome numbers (*7*, *8*), and chromosomal deviations (*9*).

The study presented here was undertaken to gain some information concerning the general validity of these observations. A number of carcinogens of high and low activity and two noncarcinogenic azo dyes were examined for their capacity to induce mitotic irregularities, to provoke a proliferation in hepatic tissue, and to lead to a heterogeneous cell population.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, weighing 180 to 200 g, were used throughout the experiments, except that in ethionine administration female rats were preferred (*10*).

Partial hepatectomies were performed according to the technique of Higgins and Anderson (*11*). The animals were killed 2 days after partial hepatectomy and then their livers removed for cytological studies.

For microscopic examinations small pieces of liver were fixed in 3 : 1 ethyl alcohol-acetic acid, processed through the usual alcohol-benzene-paraffin schedule, sectioned at 10, 15, and 20 μ, and stained by the Feulgen technique (*12*). The advantages and disadvantages of this procedure for a quantitative estimation of mitotic irregularities have been discussed (*6*).

The carcinogens and the two noncarcinogenic azo dyes were added to a 12 percent protein diet (*13*). The concentration of all these substances in the diet was 0.06 percent except ethionine, which was administered at a 0.5 percent level.

The following liver carcinogens were administered: 3′-methyl-4-dimethylaminoazobenzene [3′-Me-DAB (*14*)] and thioacetamide [TAA (*15*, *16*)] representing carcinogens of high activity; 4-monomethylaminoazobenzene [MAB (*17*)] as a sample of a moderately active carcinogen; and *o*-aminoazotoluene [AAT (*18*)], 4′-methyl-4-dimethylaminoazobenzene [4′-Me-DAB (*14*)], ethionine (*10*), and thiourea (*15*) representing substances of weak carcinogenic activity. 2-Methyl-4-dimethylaminoazobenzene [2-Me-DAB (*19*)] and 4-aminoazobenzene [AB (*14*)] were used as two azo dyes lacking the ability to induce hepatomas and cholangiomas.

The nuclear volumes were calculated from the mean nuclear diameters determined by ocular micrometry of nuclei in Feulgen-stained sections (*20–22*). The nuclei were grouped according to their ploidy on the basis of diameter measurements.

[4] *Chemical Abstracts'* nomenclature for the compounds used in this study is: *N,N*-dimethyl-*p*-phenylazoaniline = 4-dimethlyaminoazobenzene; *N,N*-dimethyl-*p*-(*m*-tolylazo)aniline = 3′-methyl-4-dimethylaminoazobenzene; *N*-methyl-*p*-phenylazoaniline = 4-monomethylaminoazobenzene; *N,N*-dimethyl-*p*-(*p*-tolylazo)aniline = 4′-methyl-4-dimethylaminoazobenzene; *N,N*-dimethyl-*p*-(4-phenylazo)aniline = 2-methyl-4-dimethylaminoazobenzene; *p*-phenylazoaniline = 4-aminoazobenzene.

We determined the mitotic rates by counting 2,000 to 2,500 nuclei for each liver sample, from random fields. Feulgen-stained preparations were used.

RESULTS

Frequency of Irregular Mitoses

Two highly carcinogenic substances, 3′-Me-DAB and TAA, one moderately active azo dye, MAB, four weakly active liver carcinogens, AAT, 4′-Me-DAB, thiourea, and ethionine, and two noncarcinogenic azo dyes, 2-Me-DAB and AB, were examined for their ability to induce chromosomal and mitotic irregularities. The method applied was the same as that used for the study of the influence of DAB on the mitotic cycle (*6*): Rats were fed a diet containing the substance in question and partial hepatectomies were performed at different time intervals to induce cell divisions necessary for the study of the effect of a given compound on mitosis.

The data in table 1 comprise the frequency of mitotic irregularities of diploid to tetraploid hepatic cells. The percentage of abnormal mitoses, in hepatic tissues of rats fed carcinogens, was in all cases considerably higher than in control rats fed a diet containing 25 or 12 percent protein, respectively. The samples were taken at random, but necrotic parts and areas with a high infiltration of leukocytes were avoided when present. Abnormal mitoses of higher polypoid cells were recorded separately and are summarized in table 2. The mitotic irregularities of these cells are around 3 times higher as compared to the diploid or tetraploid ones.

Highly polyploid cells were present in the livers of rats fed carcinogens of high activity more frequently than in animals fed a diet containing weak carcinogens or the noncarcinogenic azo dyes AB and 2-Me-DAB (table 3).

The effect on mitosis of both noncarcinogenic azo dyes examined was of particular interest. The administration of AB caused only a small rise in abnormal mitoses from the control rats fed a 25 and 12 percent protein diet (table 1). However, the frequency of mitotic irregularities induced by the noncarcinogenic azo dye, 2-Me-DAB, is relatively high, exceeding that of the weak carcinogens such as AAT, 4′-Me-DAB, thiourea, and ethionine (table 1). Highly polyploid nuclei were observed only occasionally in the livers of rats fed the noncarcinogenic azo dyes (table 3). It was therefore not possible to examine the frequency of mitotic irregularities in these highly polyploid nuclei.

The data presented in tables 1 and 2 were obtained from regenerating livers after a partial hepatectomy, which unmasked the nuclear damage produced by the carcinogens or noncarcinogenic derivatives. The question arises whether abnormal mitoses occur in the intact liver in the course of carcinogenesis. Some cell divisions must be present for study of this particular problem. An administration of 3′-Me-DAB and TAA leads to a pronounced proliferation of hepatic cells in the liver of adult

TABLE 1.—Mitotic irregularities of anaphase and telophase nuclei of diploid to tetraploid hepatic cells examined in regenerating livers after partial hepatectomy

Diet	Relative carcinogenic activity	Period of feeding (weeks)	Number of rats	Mitotic irregularities (percent)
3′-Me-DAB	12	2	4	21. 2(20. 0–23. 2)
		4	5	22. 1(19. 4–23. 7)
		8	6	26. 9(26. 7–27. 2)
TAA	12	2	7	21. 2(18. 4–23. 1)
		4	6	31. 5(31. 9–32. 7)
		8	7	23. 9(21. 1–25. 0)
		11	5	20. 6(17. 5–25. 0)
MAB	6	4	8	19. 1(16. 3–20. 9)
AAT	2	4	6	7. 72(6. 2–9. 1)
		8	5	11. 8(11. 1–13. 0)
		11	3	11. 5(10. 0–13. 0)
4′-Me-DAB	1	3	5	8. 7(5. 5–11. 5)
		8	6	11. 1(10. 1–11. 5)
Thiourea	—	3	3	8. 6(6. 9–10. 0)
		7	4	12. 0(9. 5–15. 0)
		11	10	13. 0(9. 0–17. 4)
Ethionine	—	2	5	11. 6(8. 7–14. 3)
		3	6	13. 7(11. 8–17. 0)
2-Me-DAB	0	4	5	19. 9(17. 0–23. 4)
		6	15	18. 3(13. 0–23. 8)
		11	4	15. 9(11. 1–20. 0)
AB	0	4	5	4. 8(1. 8–6. 5)
		8	5	4. 1(1. 2–6. 2)
		11	6	4. 4(3. 7–5. 1)
Controls, 25 percent protein	—	2	5	1. 1(0. 0–2. 0)
		8	5	1. 9(1. 0–2. 9)
		11	6	1. 6(1. 3–1. 9)
Controls, 12 percent protein	—	8	6	3. 2(2. 0–5. 9)

TABLE 2.—Mitotic irregularities of highly polyploid anaphase and telophase nuclei of hepatic cells examined in regenerating liver after partial hepatectomy

Diet	Relative carcinogenic activity	Period of feeding (weeks)	Number of rats	Mitotic irregularities (percent)
3′-Me-DAB	12	8	6	75. 8(68. 0–79. 5)
TAA	12	8	7	66. 6(62. 3–75. 1)
MAB	6	4	8	67. 7(60. 0–86. 7)
Ethionine	—	3	4	44. 2(40. 0–47. 5)

rats, which makes the screening for mitotic irregularities possible. The results obtained on diploid to tetraploid nuclei are shown in table 4. There is a relatively high number of mitotic irregularities, during the first mitotic wave, occurring in around 4 weeks of 3′-Me-DAB and TAA feeding. Abnormal mitoses continued to be present at all consecutive stages of carcinogenesis, the percentage remaining roughly the same.

TABLE 3.—Polyploid class frequencies in hepatic cells expressed in percentage of the total number of cells examined. Class I represents diploid nuclei

Diet	Period of feeding (weeks)	Relative carcino-genic activity	Class I 6–8*	Class II 8–10*	Class III 10–12*	Class IV 12–16*	Class V 16–19*	Class VI 19–25*	Class VII 25–*
Normal	8	—	66	31	3	0	0	0	0
3′-Me-DAB	8	12	26	21	28	10	13	1	1
TAA	8	12	66	11	9	8	5	1	0
2-Me-DAB	8	0	64	34	2	0	0	0	0
AB	8	0	59	35	6	0	0	0	0

*Diameter of interphase nuclei expressed in arbitrary units.

The results are thus comparable to those observed in rats on a DAB diet (*6*).

TABLE 4.—Mitotic irregularities of anaphase and telophase nuclei of diploid to tetraploid hepatic cells. No partial hepatectomy

Diet	Relative carcinogenic activity	Period of feeding (weeks)	Number of rats	Mitotic irregularities (percent)
3′-Me-DAB	12	4	6	20.0(16.2–22.1)
		8	8	24.3(21.4–28.0)
TAA	12	4	7	26.3(24.5–28.1)
		8	6	20.8(18.3–23.6)
		11	7	21.6(20.8–25.4)
AB	0	11	8	3.3(2.0–4.0)

An experiment was performed to reveal the persistence of the nuclear injury induced by the carcinogen 3′-Me-DAB. Adult rats were fed a diet containing 3′-Me-DAB for 4 weeks and were then returned to a normal diet. Partial hepatectomies were performed after various time intervals after the discontinuation of the carcinogen. The incidence of mitotic abnormalities in diploid hepatic cells was estimated in the regenerating liver lobe. The data, summarized in table 5, show that the percentage of mitotic irregularities did not decrease significantly even by 6 months after discontinuation of the carcinogen. These results indicate the persistence of the nuclear injury in a latent form comparable to that induced by X irradiation.

TABLE 5.—Mitotic irregularities in hepatic cells of livers regenerating after discontinuation of carcinogen

Diet	Abnormal mitoses (percent)				
	Individual cases				Average
Normal	0.0	1.4	2.0	2.1	1.3
3′-Me-DAB, 4 weeks	19.4	21.1	23.7	24.0	22.0
3′-Me-DAB, 4 weeks, and normal diet for:					
1 week	15.2	20.0	20.5	21.1	19.2
3 weeks	15.8	17.7	20.0	20.5	18.5
12 weeks	13.4	13.5	31.0	33.6	22.8
24 weeks	16.8	17.7	18.3	24.0	19.2

Morphology of Abnormal Mitosis

The carcinogens that were applied produced a variety of different mitotic irregularities. A quantitative evaluation of abnormalities at anaphase and telophase was attempted. The limitation of this procedure has been discussed (*6*). The data shown in table 6 are average values,

TABLE 6.—Different types of mitotic irregularities in hepatic cells

Abnormalities in anaphase and telophase	3′-Me-DAB		TAA		MAB	Thiourea			Ethionine		2-Me-DAB		
Weeks:	2	8	2	8	4	3	7	11	2	3	4	6	11
With acentrics alone:													
1) one acentric	35.6	50.7	42.6	52.5	47.3	79.8	73.3	49.3	68.8	48.5	66.0	48.5	40.0
2) two or more acentrics	12.6	7.0	10.0	10.5	10.5	0.0	5.2	11.5	3.1	4.7	12.0	10.0	16.7
With bridges:													
3) one bridge	26.3	23.1	33.0	15.4	33.0	9.5	13.0	22.9	18.5	29.0	6.0	12.8	16.7
4) two bridges	9.6	10.5	9.0	3.9	3.7	0.0	3.3	5.3	9.6	6.5	2.0	6.5	3.3
5) with bridges and acentrics	6.5	6.8	2.9	10.8	4.6	10.7	5.2	10.0	0.0	11.3	14.0	19.2	20.0
Multipolar mitosis	9.4	1.6	1.9	6.7	1.2	0.0	0.0	0.9	0.0	0.0	0.0	2.9	3.3

each obtained from 1,000 to 1,200 abnormal anaphase-telophase nuclei pooled from 5 rats. They are expressed in percentage of the total number of abnormalities to facilitate a comparison. The data comprise diploid, polyploid, and aneuploid nuclei. Samples of various irregularities at anaphase and telophase found in the precancerous liver of a TAA-fed rat are given in figures 3 to 13 and 15. A normal diploid cell at telophase is shown in figure 14. By a comparison of the type of mitotic abnormalities produced by different liver carcinogens, by eggs of *Taenia crassicollis* (*23*) and by X rays (*24*, *25*) a striking similarity becomes evident.

A number of different abnormalities involving the mitotic apparatus were present in the precancerous livers. Tripolar and tetrapolar mitosis can be placed in this category (figs. 1, 9, 10, and 15). They might lead to multinucleated cells consistently found in livers of rats fed active carcinogens (figs. 16 and 17). A second cause of multinucleated cells can be sought in the pronounced shortening of the spindle fibers resulting in an incomplete separation of anaphase and telophase nuclei (figs. 12 and 13). This aberration is similar to that found in livers of rats fed a diet containing DAB (*6*).

Abnormalities at the metaphase stage are more difficult to evaluate for reasons mentioned previously (*6*). A quantitative estimation was therefore not attempted. A shift in the ratio of metaphase to anaphase and telophase nuclei was an irregularity produced by all carcinogenic substances. The extent of this shift, however, varied greatly (table 7). The noncarcinogenic azo dye AB lacked the ability to interfere with the metaphase-to-anaphase and telophase ratios, whereas 2-Me-DAB, though noncarcinogenic, caused this shift.

The fate of abnormal metaphase nuclei is difficult to evaluate. Some of them will probably proceed and complete a mitotic cycle. Others will become pyknotic and finally disintegrate. The stickiness and clumping of chromosomes shown in figure 2 are apparently the first stages of this process, leading to the formation of micronuclei and chromosome clumps, illustrated in figure 18. The possibility cannot be neglected that some of the metaphase nuclei will revert directly into interphase nuclei. This process might represent one way of producing polyploid cells.

DISCUSSION

Hepatomas of rats and man consist of cells with altered genotypes as shown by an abnormal content of deoxyribonucleic acid (*6*), aneuploid chromosome numbers (*7*, *8*), and new chromosome combinations (*9*). These observations raised the question as to the mechanism leading to aneuploid genotypes. A cytological examination of precancerous lesions of the liver induced by DAB revealed the presence of high numbers of abnormal mitoses resulting in the formation of a genetically heterogeneous cell population. Thus an opportunity is provided for a selection of particular cell lines, some of them giving rise to hepatomas (*6*). These

TABLE 7.—Metaphase-to-anaphase and telophase ratios in hepatic cells

Diet	Relative carcinogenic activity	Period of feeding (weeks)	Number of rats	Ratio metaphases: anaphases+telophases
3′-Me-DAB	12	4	5	0.9 (0.8–1.2)
		8	6	1.6 (1.4–1.7)
		11	6	0.9 (0.8–0.9)
TAA	12	4	6	0.9 (0.8–0.9)
		8	7	1.1 (1.1–1.2)
		11	5	1.5 (1.1–1.7)
MAB	6	4	8	1.4 (1.2–1.4)
AAT	2	4	6	1.3 (0.9–1.9)
		8	5	1.7 (1.4–1.8)
		11	3	1.8 (1.8–1.9)
4′-Me-DAB	1	3	5	1.1 (0.8–1.4)
		8	6	1.6 (1.0–1.8)
Thiourea	—	3	3	1.0 (0.8–1.0)
		7	4	1.2 (1.1–1.5)
		11	10	1.3 (1.1–1.7)
Ethionine	—	2	5	1.3 (1.2–1.4)
		3	6	1.5 (1.1–1.8)
2-Me-DAB	0	4	5	1.7 (1.6–1.8)
		6	15	1.3 (1.1–1.7)
		11	4	0.7 (0.5–1.0)
AB	0	8	5	0.6 (0.6–0.8)
		11	6	1.0 (0.7–1.0)
Control	—	4	6	1.0 (1.0–1.1)
		8	5	1.1 (1.0–1.1)
		11	6	1.0 (1.0–1.2)

results lead to the conclusion that one essential effect of a carcinogenic substance is the induction of mitotic irregularities.

The general validity of this conclusion was tested by a comparison of the effect of liver carcinogens of high and low carcinogenic activity and of two noncarcinogenic azo dyes on the mitotic cycle. All the liver carcinogens so far examined had the ability to induce a relatively high number of mitotic irregularities in hepatic cells of regenerating liver.

The types of mitotic abnormalities varied greatly, and ranged from chromosome damage to incomplete spindle function. This broad spectrum of mitotic irregularities was found in all precancerous livers regardless of the carcinogen used. Eggs of *Taenia crassicollis*, which induce hepatomas, produced a relatively high incidence of abnormal mitoses (*23*) similar to that produced by chemical compounds that are liver carcinogens.

The question arises whether the production of mitotic irregularities can be considered a common effect of liver carcinogens. The lack of noncarcinogenic AB to induce any significant number of abnormal mitoses is in accord with this assumption. 2-Me-DAB, although noncarcinogenic, is able to produce a relatively high percentage of mitotic irregularities in

the regenerating liver. Why then does a 2-Me-DAB application not lead to the formation of hepatomas?

It is well known that a loss of chromosomes or chromosome fragments and an unequal distribution of chromosomes to both daughter nuclei take place only during a mitotic cycle. A proliferation is therefore mandatory to obtain cells with abnormal chromosome complements. An instructive example is the effect of X rays on the liver of fully grown rats. X irradiation induces a chromosome injury in hepatic cells, which is revealed only when these cells are induced to divide after partial hepatectomy (*24*, *25*) or a carbon tetrachloride application (*26*). A given substance must be able to induce chromosome abnormalities and, in addition, a proliferation to cause the formation of cells having an abnormal genotype.

All tested substances which are liver carcinogens are able to induce mitotic irregularities, as shown previously, and are able to provoke a proliferation in the livers of adult rats (*27–30*). The noncarcinogenic 2-Me-DAB lacks the ability to induce mitoses in hepatic tissue. An application of this azo dye will not, therefore, produce any genetically abnormal cells despite its capability of inducing a chromosome injury, as revealed in the regenerating liver after partial hepatectomy. The noncarcinogenic azo dye AB lacks the ability to induce mitotic irregularities as well as a proliferation.

The effect of liver carcinogens is comparable to that of X rays. Both are able to produce a nuclear injury in the intermitotic cell which can persist over a long period in a latent form (*24–26*). Chemicals having this property are usually referred to as "radiomimetic substances" (*31*). There is, however, a difference between the effect of X rays on hepatic tissue and that of a liver carcinogen. Only the carcinogens are able to induce a proliferation in the livers of adult rats.

The data presented seem to justify the conclusion that both factors, cell proliferation and mitotic irregularities, must be present to lead to the formation of hepatomas. The presence of a nuclear injury without proliferation, as in 2-Me-DAB application or irradiation, as well as a proliferation without the presence of a relatively high incidence of mitotic irregularities, as in regenerating liver after partial hepatectomy, are apparently insufficient to transform a normal cell into a malignant one. The results support the assumption that chromosomal alterations are involved in the formation of neoplastic cells (*32*, *33*). The mitotic irregularities present in preneoplastic lesion can be considered as the source producing the raw material necessary for selection of neoplastic cells containing particular abnormal genotypes.

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PLATES

PLATE 206

Microphotographs of hepatic cells from rats fed a 3′-Me-DAB diet for 8 weeks (Feulgen stain).

FIGURE 1.—Tetrapolar polyploid metaphase.

FIGURE 2.—Arrested polyploid metaphase with clumped chromosomes.

FIGURE 3.—Telophase with chromosome fragments.

FIGURE 4.—Anaphase with chromosome fragments.

FIGURE 5.—Highly polyploid late anaphase with chromosome fragments.

FIGURE 6.—Diploid anaphase with chromosome bridge.

FIGURE 7.—Octaploid telophase with two chromosome bridges and one aberrant chromosome or chromosome fragment.

FIGURE 8.—Sixty-four-ploid telophase with chromosome bridge and chromosome fragments.

FIGURE 9.—A tripolar mitosis with chromosome bridges and chromosome fragments.

FIGURE 10.—A tetrapolar mitosis with chromosome bridges and chromosome fragments.

FIGURE 11.—Tripolar mitosis of a diploid nucleus.

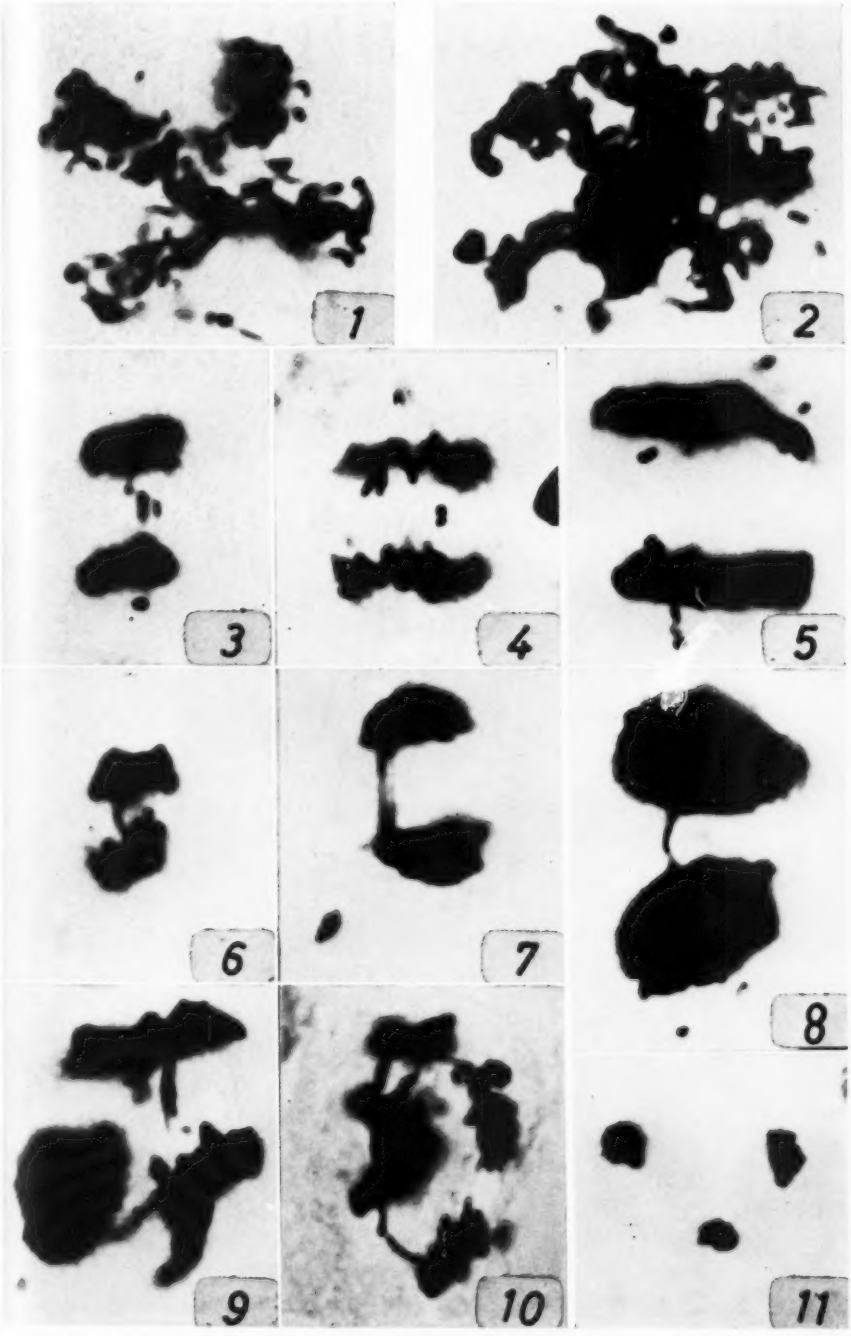
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PLATE 207

Microphotographs of hepatic cells from rats fed a 3′-Me-DAB diet for 8 weeks (Feulgen stain).

FIGURE 12.—Incompletely separated telophase nuclei.

FIGURE 13.—Incompletely separated telophase nuclei.

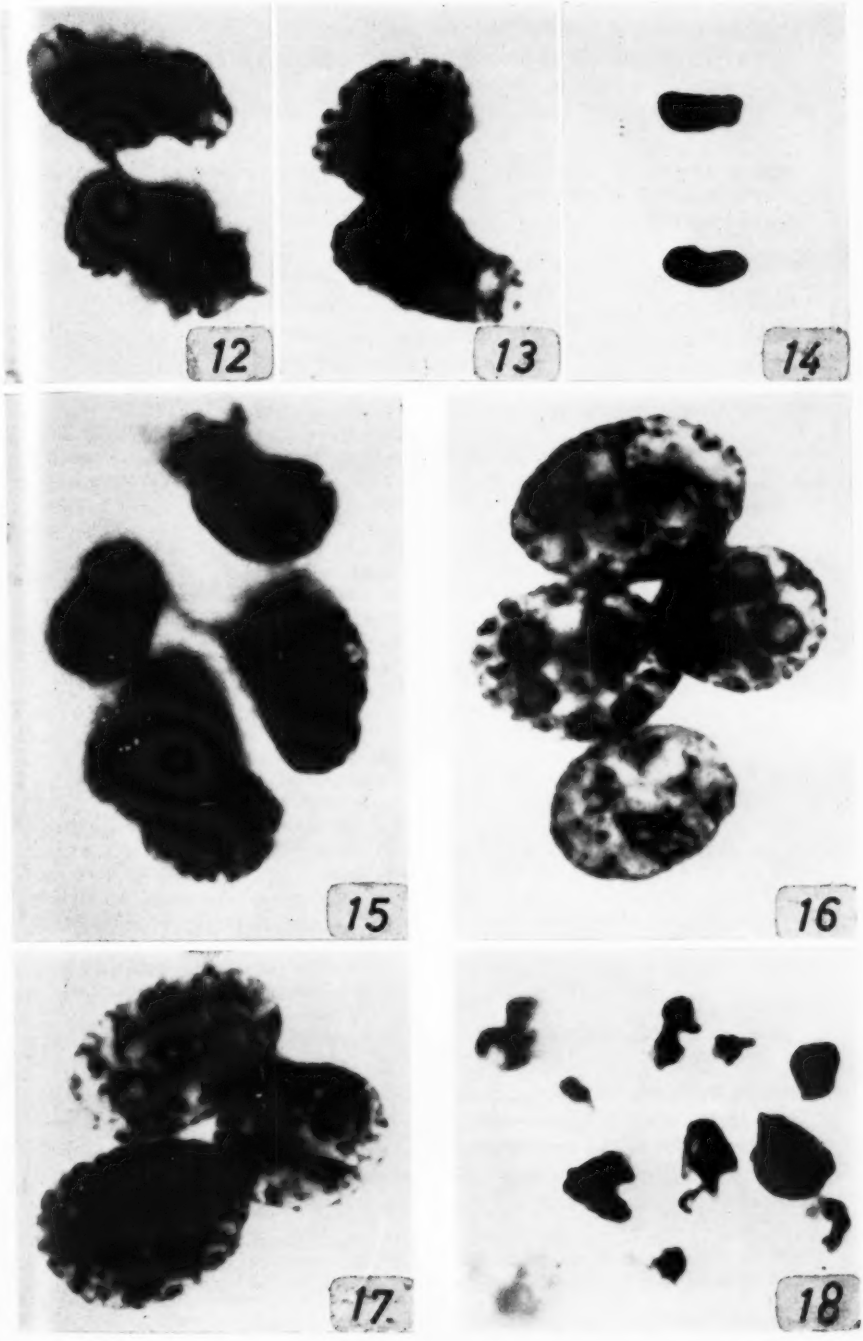
FIGURE 14.—Diploid telophase of a hepatic cell from normal liver.

FIGURE 15.—Telophase plates of unequal size of tetrapolar mitosis (around 128-ploid).

FIGURE 16.—Tetranucleated cell.

FIGURE 17.—Trinucleated cell.

FIGURE 18.—Pyknotic "micronuclei."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Observations on Inclusion Bodies in Normal and Pathologic Mucosa of the Colon in Man [1, 2]

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SUMMARY

Specimens of histologically normal and pathologic colonic mucosa in man with diseases such as ulcerative colitis, adenomatous polyps, carcinoma, acute and chronic diverticulitis, and acute suppurative appendicitis revealed similar spheroidal bodies demonstrable with the Feulgen's technique. These bodies were found intracellularly in epithelial cells and extracellularly in the adjacent lamina propria or stroma and in luminal material. They were localized mostly in cytoplasm but in certain cases were also found within nuclei. The presence of inclusion bodies in benign and malignant intestinal polyps and in ulcerative colitis has been previously reported by others, who suggested that they are evidence of the viral nature of these diseases. The reactivity of inclusion bodies with the Feulgen's technique is merely cytochemical evidence that they contain deoxyribonucleic acid (DNA). Since this technique does not indicate the origin and pathway of formation of the DNA shown in inclusion bodies, more factual evidence is required to support the stimulating hypotheses that they are cytologic evidence of the viral nature of intestinal polyps and ulcerative colitis. Inclusion bodies were found in 66 percent of the cases of ulcerative colitis in this study. The incidence of carcinoma in patients with ulcerative colitis is 3 to 6 percent in various series. Therefore, the suggested relationship between ulcerative colitis and intestinal polyps and carcinoma, on the basis of the finding of inclusion bodies in all these conditions, seems not to be supported by the correlation frequency of inclusion bodies and incidence of carcinoma in ulcerative colitis. It is suggested that degenerating cell processes and migration of leukocytes through the epithelium of the intestinal mucosa may account for the particles of DNA in the intestinal mucosa of man reported in this and other studies.—J. Nat. Cancer Inst. 26:1429–1443, 1961.

IT HAS been reported (*1*, *2*) that inclusion-like bodies are demonstrable with Feulgen's technique in the cytoplasm of epithelial cells in specimens of early and chronic ulcerative colitis. These bodies containing deoxyribonucleic acid (DNA) were interpreted as being of possible viral nature and

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similar to the inclusion bodies previously described in human rectal polyps (*3*).

This report deals with a study of normal and pathologic intestinal mucosa and includes specimens of nonspecific ulcerative colitis to establish the frequency and distribution of spheroidal particles stainable with Feulgen's technique. It will be shown that particles reactive with this technique and with the appearance previously described (*1–3*) can also be found in normal intestinal mucosa and various other pathologic conditions (*4*).

MATERIALS AND METHODS

Tissue blocks of normal and pathologic colonic mucosa were obtained from surgical specimens and endoscopic biopsies. The series included 18 specimens of normal colonic mucosa, 21 of ulcerative colitis, and 21 from patients with various pathologic conditions of the colon, of which there were 3 specimens each from acute suppurative appendicitis and acute and chronic diverticulitis of the sigmoid colon, 5 from benign adenomatous polyps, some of which showed dedifferentiation, 9 from adenocarcinoma of the colon, 1 from postradiation proctitis, and 1 of normal secretory endometrium. Formalin- or Helly-fixed, paraffin-embedded tissue sections were stained with Feulgen's technique and its corresponding control, the direct Schiff procedure in which acid hydrolysis is omitted (*5*). In all the cases, sections with and without counterstain with Fast Green FCF were examined. Other sections were stained with hematoxylin and eosin.

RESULTS

Spheroidal bodies demonstrable with the Feulgen's technique were found in a certain number of specimens of normal colonic mucosa and in the pathologic tissues studied (table 1). The number of bodies in each case was largest in carcinoma and in benign adenomatous polyps. In the last condition, bodies were encountered in larger number in areas showing dedifferentiation. Normal mucosa showed the smallest number of Feulgen-positive particles. Specimens from acute and chronic inflammatory conditions, such as diverticulitis and appendicitis, contained more bodies than did normal mucosa, but less than specimens from patients with ulcerative colitis, which contained approximately the number of bodies demonstrable in neoplastic proliferating lesions. The spheroidal bodies were located within epithelial cells and extracellularly in the stroma. The intracellular bodies were usually intracytoplasmic (figs. 1 through 8, 12, and 14 through 16), but in certain conditions, *e.g.*, carcinoma, adenomatous polyps, and ulcerative colitis, intranuclear bodies were also found (figs. 10 through 13). In some of these, the nuclei appeared enlarged or the normal pattern of Feulgen-positive substances in these cells altered (fig. 11). The nuclear membrane was faintly stained in some

nuclei in which clumping of chromatin material depicted spheroidal bodies (figs. 11 and 13). The size, appearance, and number of inclusion bodies found correspond to previous reports (*1–3*). In certain cases, notably in adenomatous polyps, bodies were irregular in shape. In addition, spheroidal bodies were also found in the stroma (figs. 3 and 4) and in the lumen of glandlike structures, sometimes within desquamated and degenerating cells, or in the midst of mucinous substances in neoplasms (fig. 5), in the inflamed lamina propria of the intestinal mucosa in ulcerative colitis, and in the other inflammatory conditions studied (figs. 7 and 9). Lymphocytes and neutrophils were in or between epithelial cells of the mucosa of the various tissues studied (figs. 1 and 2). This is morphologic evidence of migration of leukocytes through the intestinal mucosa. Plasma cells showed, in some cases, peculiar clumping of chromatin which resembled the spheroidal bodies (fig. 11). Eight of the 18 specimens of normal colonic mucosa showed inclusion bodies (fig. 15). Fourteen of the 21 specimens of ulcerative colitis contained spheroidal bodies of various sizes in the cytoplasm and occasionally within the nuclei of cells of the surface epithelium and crypts of Lieberkühn (figs. 1, 2, and 10). All 9 adenocarcinomas of the colon (figs. 4, 5, and 13) and the 3 adenomatous polyps (figs. 3 and 12) had abundant spheroidal particles. The 3 specimens of acute suppurative appendicitis (figs. 8 and 9) and 2 of the 3 with diverticulitis (figs. 6 and 7) contained inclusion bodies. In all, the reactive particles were frequently surrounded by a clear halo. For purpose of comparison, a section of endometrial mucosa is included as an example of another normal tissue in which inclusion bodies are also found (fig. 16).

TABLE 1.—Distribution of Feulgen-positive spheroidal bodies in various conditions

Conditions	Positive	Negative	Total
Normal colon and rectum	8	10	18
Ulcerative colitis	14	7	21
Adenocarcinoma of colon	9	0	9
Adenomatous polyp of colon	3	0	3
Diverticulitis of colon (acute and chronic)	2	1	3
Acute suppurative appendicitis	3	0	3

DISCUSSION

Cytoplasmic inclusion bodies containing DNA were reported to be present in cells of human rectal polyps (*3*). Since they were not found in a control series of tissues, it was concluded that the finding of those bodies was suggestive evidence of the viral nature of benign and malignant human rectal polyps. Reports of spheroidal bodies in specimens of ulcerative colitis, which resemble those previously described in intestinal polyps, have been published (*1*, *2*). Although no attempt was made to establish their presence in normal and other pathologic tissues, the inference was nevertheless drawn that a virus may be etiologically related to ulcerative colitis.

Our observations indicate that inclusion bodies which can be demonstrable by Feulgen's technique may also be seen in normal colonic mucosa and other pathologic tissues of the intestine, as well as in those conditions previously reported.

There is general agreement that reactivity of a cytologic constituent with the Feulgen procedure, coupled with a negative result with the direct Schiff procedure, is histochemical evidence that it contains a nucleic acid of the deoxypentose type (*6*), but obviously does not indicate the origin and pathway of formation of the reactive substance. It is known that viruses contain nucleoproteins (*7*, *8*). Although many inclusion bodies of viral diseases are eosinophilic and Feulgen-negative, this is not necessarily an indication that they are entirely devoid of deoxyribonucleoproteins, since the amount present may be below the threshold of sensitivity of the Feulgen technique. Supporting this assumption is the study of inclusion bodies of vaccinia and their relationship to elementary bodies observed in tissue cultures of the rabbit's corneal epithelium (*9*). It was shown that these cytoplasmic elementary bodies are negative with the Feulgen procedure; only when they become enlarged do they react. This possibly indicates that higher concentrations of DNA render the reaction positive. Elementary bodies studied in the test tube were positive with the Feulgen technique, whereas films of these elementary bodies on coverslips were negative (*10*).

Intracellular inclusion bodies have been found in virus diseases, in certain conditions which are not considered of viral nature, and in apparently healthy human tissues [(*11–13*), figs. 15 and 16]. Yet they have been regarded as the most characteristic histologic evidence of viral infection (*14*), despite the fact that inclusion bodies are not a constant finding in viral diseases. "Only about half of the sixty known or suspected virus diseases of man are associated with the development of inclusion bodies" (*15*, *16*). Some of these bodies are clusters of virus. Although certain inclusion bodies contain elementary bodies assumed to constitute the virus itself, such as in fowlpox, lymphogranuloma venereum, and molluscum contagiosum, it has been suggested that it may be erroneous to assume that all inclusion bodies have a similar structure (*17*).

The finding of intranuclear inclusion bodies in virus diseases attracted attention long ago, and careful studies of their appearance, distribution, and significance have been made and their classification attempted (*18*). But the list of conditions, in which intranuclear inclusion bodies have been found and in which no virus is held responsible, is long (*11*). Even physical and chemical agents can produce them. Thus, a certain number of cases of lead poisoning in children showed intranuclear inclusion bodies in the tubular epithelium of the kidney and in hepatic cells (*19*). In animals they have been shown in various normal states and under experimental conditions: in mice treated with thioacetamide, azo dyes, or fed special diets (*20*). In various reports intranuclear inclusion bodies have also been shown in tumors (*11*).

Cytoplasmic inclusion bodies are characteristic of some viral diseases

such as smallpox, vaccinia, and molluscum contagiosum (*11*). Their mechanisms of formation and structure are far from understood. Two examples of various experimental approaches leading to a more thorough evaluation of the significance of inclusion bodies may be mentioned: 1) The relationship of the herpes virus to the cell nucleus has been subjected to various interpretations. The significance of the inclusion bodies and their relationship to the herpes virus (*21*) have been greatly clarified by the use of cytochemical techniques which showed that variations in appearance and reactivity of the inclusion bodies with the Feulgen procedure (*22*) parallel the variations in content of viral antigen of these bodies (*23*). Thus, when no viral antigen was demonstrable with the fluorescent technique, the Feulgen's reaction became negative. 2) Inclusion bodies, studied by electron microscopy in the hepatic cells of mice receiving colchicine and in the Crocker ascites tumor cells, were shown to be actually cytoplasmic substance *included* within the nucleus. These bodies communicated with the surrounding cytoplasm through wide channels limited by a double membrane which is part of the nuclear membrane (*24*). These observations are consistent with studies showing that intranuclear inclusion bodies in the hepatic cells of mice fed a methionine-rich basal/bentonite diet, as well as inclusions which occur spontaneously in a transplantable hepatoma, contain materials of cytoplasmic origin limited by a Feulgen-positive membrane which have become segregated within the nucleus (*20*). In the same study, the real complexity of these structures is shown by the finding that they also contain enzymes which normally are only demonstrable in the cytoplasm.

The significance of Feulgen-positive inclusion bodies in polyps has been previously discussed by Leuchtenberger. The possibility that such bodies may be related to nuclear debris of necrobiotic epithelial or white cells was considered and then rejected (*1*). The same reasoning was the basis for the assumption that inclusion bodies found in ulcerative colitis were possibly of viral nature (*2*, *3*). Although desquamated or degenerated cells contain pyknotic nuclei (*25*) indistinguishable from the DNA-containing particles in cells, stroma, and luminal material, it is not possible in this present study to ascertain the identity or relationship of both groups of cellular structures.

It should be mentioned that after irradiation small clusters of spherical particles will appear in certain tissues. These bodies stain with hematoxylin and the Feulgen's procedure, and absorb ultraviolet light intensely at 263 mμ (*26*, *27*). It has been shown in mice receiving intraperitoneal injections of tritiated thymidine and subsequently irradiated that clusters of labeled debris in the intestinal mucosa were in large proportion derived from previously labeled cells (*27*).

DNA-containing bodies have been found in human peroral duodenal and jejunal biopsies after antifolic therapy with methotrexate and dichloromethotrexate. Optical and electron microscopic studies of these structures in no way suggest that they are composed of viruses (*28*).

The possibility that the inclusion bodies found in this study may also

be related to white cells migrating through the mucous membrane (figs. 1 and 2) can be proposed as a working hypothesis (*29*, *30*). There is no apparent explanation for the variation in number of inclusion bodies found in the various conditions studied. While varying rates of cell proliferation and degeneration may be suggested, no such explanation finds conclusive support. It is very suggestive that large number of Feulgen-positive spheroidal particles were found in epithelial cells and in the stroma of endometrial mucosa in late stages of the secretory phase (fig. 16).

The claim that the bodies shown in polyps and ulcerative colitis resemble inclusion bodies containing virus units, such as in molluscum contagiosum and verruca vulgaris (*1–3*), seems unwarranted, if attention is also paid to all the cytochemical changes occurring in the host cells (*31*). Yet the demonstration in this study by means of the Feulgen's technique of nucleoprotein-containing bodies in diseased and normal intestinal mucosa does not invalidate the assumption that they all may be of viral nature. This is in keeping with a growing trend to look for viruses as causative agents of neoplastic proliferations (*32*). There has been full realization of the difficulties inherent in the design of experiments to test for such a causative agent (*33*). Nevertheless, recently attention has been called again to the need for the fulfillment of Koch's basic postulates to ascertain the role of virus in producing tumors (*34*). None of the virus or virus-like agents responsible for neoplastic proliferation in mammals has thus far been propagated in chick-embryo cells (*33*).

The suggested relationship between ulcerative colitis and intestinal polyps and carcinoma on the basis of the finding of inclusion bodies (*2*, *3*) in all these conditions seems not to be supported by the correlation frequency of inclusion bodies in various processes and the incidence of carcinoma in ulcerative colitis. Thus, while inclusion bodies were found in 66 percent of the cases of ulcerative colitis in this study, the incidence of carcinoma in patients affected with ulcerative colitis is 3 to 6 percent in various series (*35–37*). Pathologic processes other than ulcerative colitis, in which large numbers of inclusion bodies were also found, are not related to carcinoma. At the present time factual evidence of the viral nature of the inclusion bodies in normal and pathologic intestinal mucosa is lacking. Further studies that will lead to a better understanding of the significance of inclusion bodies found in these conditions are desirable. Meanwhile, the origin, significance, and fate of particles containing DNA present in normal and pathologic intestinal mucosa remain unknown.

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PLATE 208

***Arrows* indicate presence of Feulgen-positive spheroidal particles. Those which are intracellular are called inclusion bodies.**

FIGURE 1.—Ulcerative colitis. Mucosa of colon. Feulgen's technique, no counterstain. Several inclusion bodies are located at basal aspect of epithelial cells underlying nuclei. Mitotic figure (M) can also be seen. In addition, neutrophils (L), some of which show pyknotic nuclei, appear in or between epithelial cells—morphologic evidence of migration of leukocytes through the mucous membrane. Lamina propria show increased cellularity. There are neutrophils, lymphocytes, histocytes, and eosinophils. × 850

FIGURE 2.—Ulcerative colitis. Colonic mucosa. Feulgen's technique, no counterstain. A crypt of Lieberkühn contains deeply stained inclusion bodies located in cytoplasm of epithelial cells. *Note* their random distribution and variation of size and shape. At base of this crypt 2 cells show deeply stained nuclei (L). They presumably are lymphocytes. Columnar cells (C) in adjacent crypt at *left* show chromatolysis. × 850

FIGURE 3.—Benign adenomatous polyp of colon, showing dedifferentiation. Feulgen's technique, no counterstain. There are numerous bodies, some of which are of irregular shape, mostly concentrated at basal aspect of epithelial cells. Some particles (1 and 2) are in stroma. × 850

FIGURE 4.—Infiltrating adenocarcinoma of colon. Feulgen's technique, no counterstain. There are several spheroidal particles. *Note* halo surrounding some of them (H). × 850

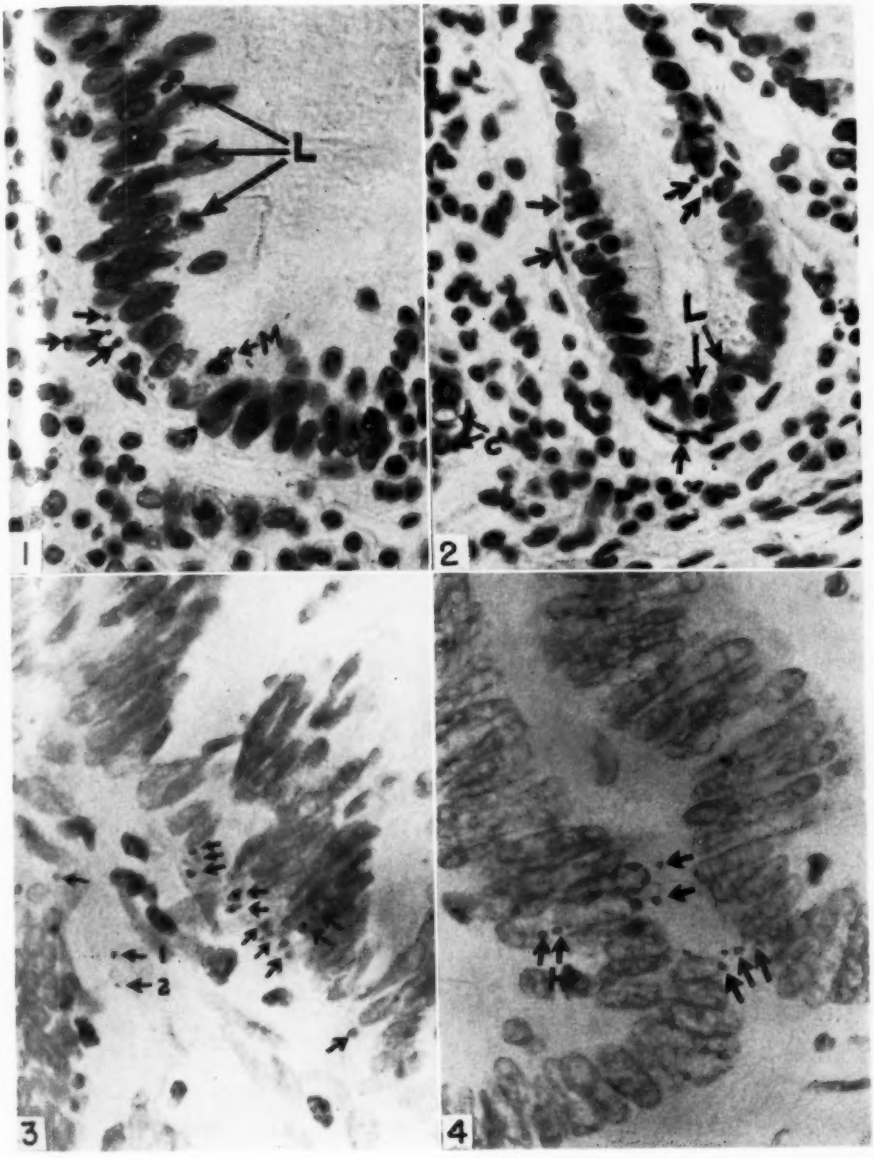

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PLATE 209

FIGURE 5.—Adenocarcinoma of colon. Feulgen's technique, with counterstain. Intensely reactive spheroidal bodies of various sizes are shown in lumen of ductlike structure. They are nuclear fragments originating in degenerating cells. × 850

FIGURE 6.—Chronic diverticulitis of sigmoid colon (case 1). Feulgen's technique, with counterstain. Covering epithelial cells show 2 inclusion bodies. There is a halo surrounding largest body. × 850

FIGURE 7.—Diverticulosis of colon with acute and chronic diverticulitis (case 2). Feulgen's technique, with counterstain. Spheroidal bodies are seen in lamina propria. A ribbon of epithelial cell became detached from lamina propria; this is an artifact. × 850

FIGURE 8.—Acute suppurative appendicitis (case 1). Feulgen's technique, with counterstain. A crypt of Lieberkühn. There are several inclusion bodies of various sizes. Normal histologic structure is altered by presence of inflammatory cells. × 850

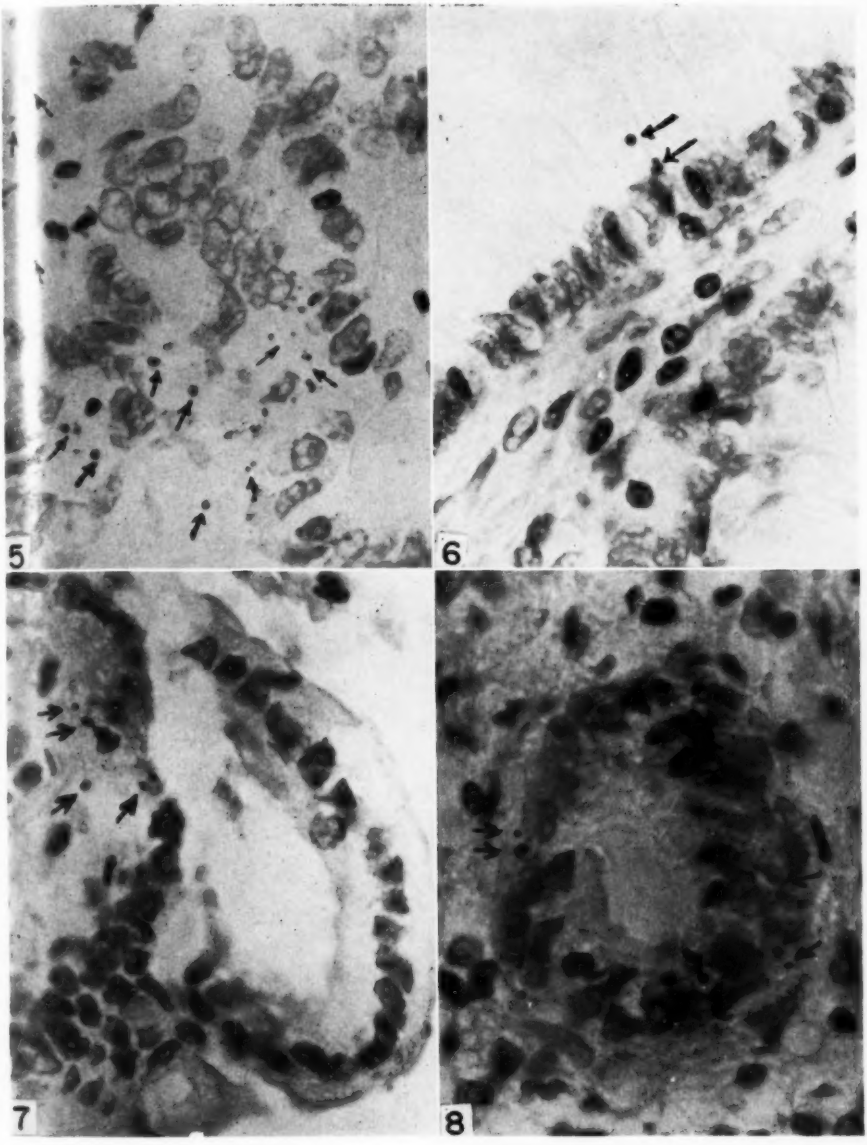
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PLATE 210

FIGURE 9.—Acute suppurative appendicitis. Feulgen's technique, with counterstain (case 2). Inflamed lamina propria shows several spheroidal particles of various sizes, the appearance of some of which (P) is very suggestive of pyknotic changes. Halo is shown in some of these (H). × 850

FIGURE 10.—Ulcerative colitis. Feulgen's technique, no counterstain. A cell (E) in a crypt. Chromatin is clumped in 2 bodies of various sizes. The larger shows incomplete but definite halo. Circular area in which the bodies are contained is faintly positive which indicates that the entire structure contains DNA. There is distortion of vicinal nuclei. Lamina propria is inflamed. × 850

FIGURE 11.—Ulcerative colitis. Colonic mucosa. Feulgen's technique, no counterstain. An area of lamina propria of mucosa containing plasma-cell nuclei (P). Two cells (P^1) show masses of chromatin which are spheroidal in shape. Nuclear membrane is seen. Compare with figure 13. × 1,700

FIGURE 12.—Benign adenomatous polyp showing dedifferentiation. Feulgen's technique, no counterstain. Spheroidal bodies of various sizes are stained. Largest is surrounded by a vacuole partially limited by a Feulgen-positive membrane (M) suggesting that it may be intranuclear. × 850

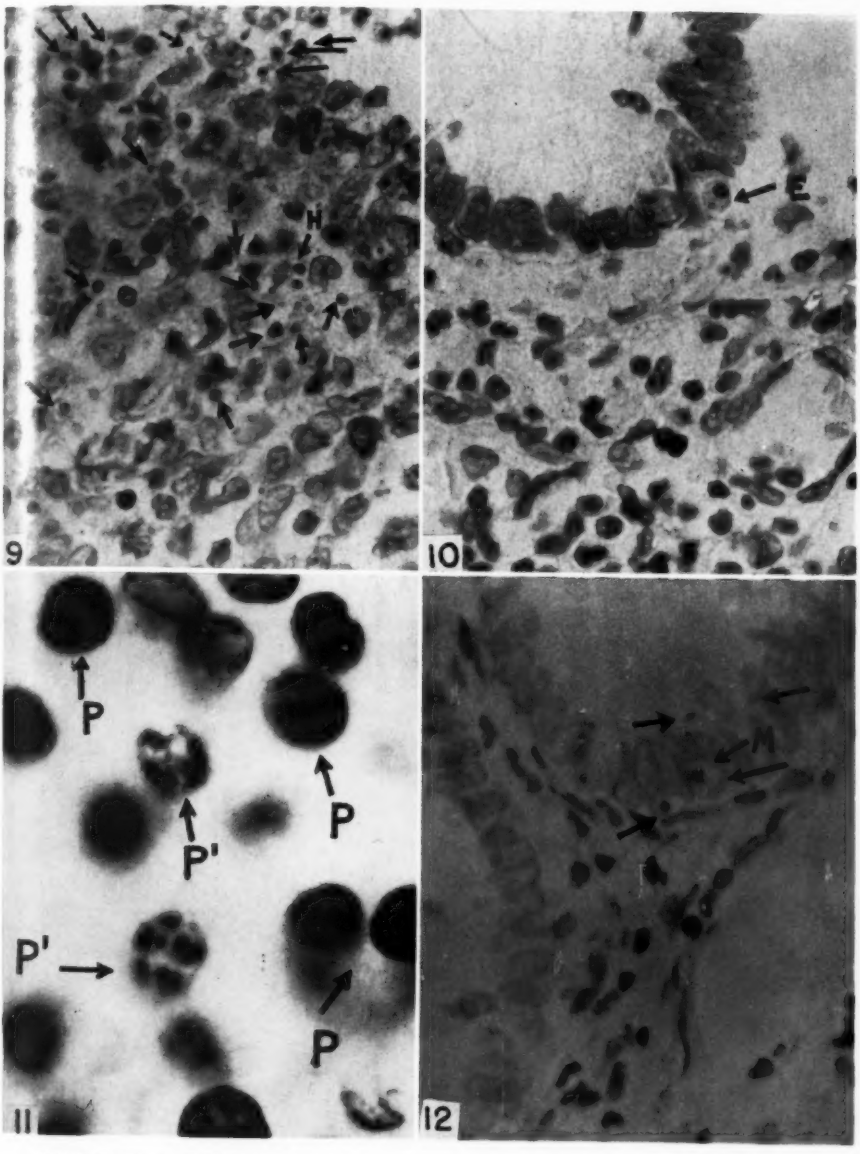

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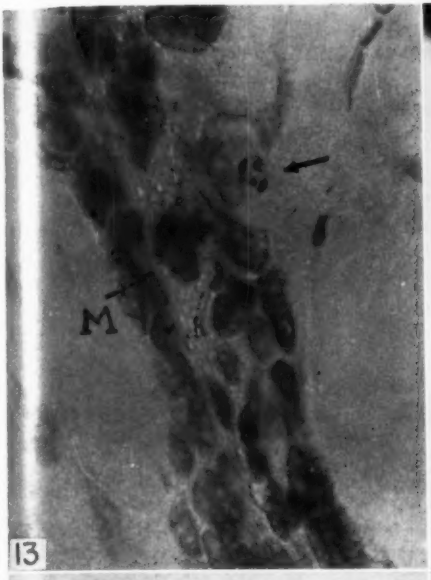
PLATE 211

FIGURE 13.—Infiltrating adenocarcinoma. Feulgen's technique, with counterstain. Nucleus located *above* and to *right* of a tripolar mitosis (M) shows clumping of chromatin depicting bodies which resemble those found in cytoplasm, stroma, and luminal material in other areas of same section. Partially limiting nuclear membrane is still faintly seen. Compare with figure 11. × 850

FIGURE 14.—Postradiation proctitis. Feulgen's technique, with counterstain. Several bodies are shown in epithelial cells. × 1,700

FIGURE 15.—Biopsy of rectum. No pathologic findings. Feulgen's technique, no counterstain. There are 2 inclusion bodies which are deeply stained in the basal aspect of epithelial cells. Both show a halo which is more apparent in one to *left*. Section stained with hematoxylin and eosin showed a normal mucosa. × 850

FIGURE 16.—Endometrial mucosa. Feulgen's technique, with counterstain. Glands in a secretory phase. There are numerous intensely reactive particles. Their origin in karyorrhectic nuclei is shown in some of them (N). × 850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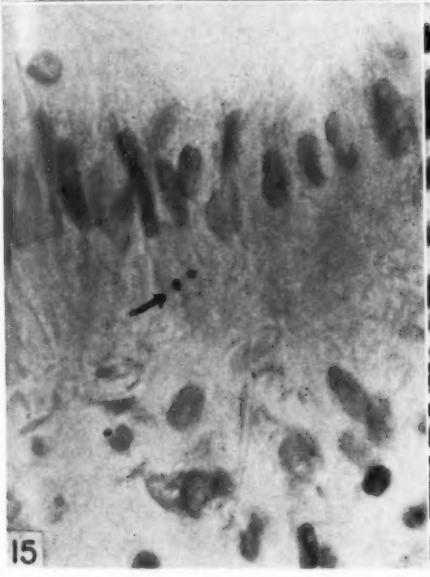
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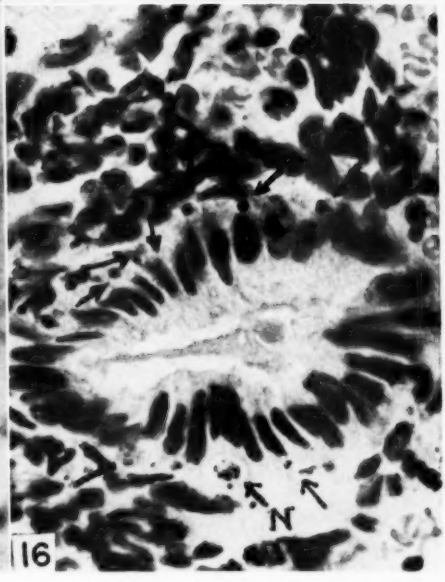
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Epithelial and Stromal Roles in Carcinogenesis. II. Tumor Development in Internal Transplants of Skin and Epithelium Treated With 3-Methylcholanthrene [1,2]

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SUMMARY

The site of action of chemical carcinogens was studied by painting the skin with 3-methylcholanthrene, followed by separate transplantation of the epithelium and stroma from the painted areas. Full-thickness skin was similarly transplanted after carcinogen application. All transplants were placed internally, which thus avoided the uncertainty of the origin of the epithelium occasioned by the ingrowth of neighboring epithelium which occurs when skin is transplanted to external sites. Normal skin and fully developed tumors survived and proliferated after transplantation. Tumors did not develop in the transplants of pure epithelium. Few tumors developed in transplants of full-thickness skin, unless tumors were already present in the skin before it was transplanted. Tumors did develop in the painted sites from which transplants were taken. Experiments have eliminated the strain of mice used, the transplantation site, and transplant necrosis as primary factors in the failure of transplants to develop tumors. Failure was not due to inhibiting factors in the host caused by exposure to carcinogen or by the growth of tumors in the areas painted, since no tumors developed in transplants made to normal mice of the same strain. It was concluded that one of the ultimate steps in carcinogenesis was inhibited by transplantation.—J. Nat. Cancer Inst. 26: 1445-1459, 1961.

THIS STUDY was undertaken to determine whether 3-methylcholanthrene applied to the skin produces epithelial tumors by a direct action on the epithelium or by a primary effect on the supporting connective tissue. Histologic changes are present in the connective-tissue stroma of precancerous skin conditions (senile elastosis and kraurosis vulvae) and after the application of both X ray and chemical carcinogens. These stromal changes may provide the stimulus for the development of epithelial tumors (*1*). To test this hypothesis, an investigation was made of the incidence of tumors in transplants of pure epithelium and in transplants

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[4] Affiliated with Columbia University.

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of full-thickness skin taken from painted areas before tumor development. Transplants were placed in internal sites (subcutaneous and intraperitoneal) to facilitate the subsequent assessment of viability and tumor formation (*2*). Control observations were made in all experiments of concurrent tumor formation in the painted areas from which the transplants were taken. In some experiments similarly painted mice, not subjected to operation, served as additional controls.

MATERIALS AND METHODS

Female mice of strains A, C3H, C57BL, and BALB/c, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were received when 4 to 6 weeks of age. They were kept in groups of 30 in colony cages during the period of painting with carcinogen and in individual cages thereafter. Mice were fed a balanced diet [5] and water *ad libitum*. They were painted with a 0.6 percent solution of 3-methylcholanthrene in benzene. The solution was kept in an amber bottle in the dark. In early experiments some variation in tumor incidence and time of tumor appearance could be attributed to deterioration of the 3-methylcholanthrene solution. Fresh solution was prepared at the onset of each later experiment. Mice were painted on either the ears or the back 3 times a week, with a single stroke of a #3 camel's-hair brush dipped once in 3-methylcholanthrene solution. An optimal total exposure to 3-methylcholanthrene was sought which would permit transplantation of the painted skin before tumors appeared. Furthermore, a sufficient exposure to 3-methylcholanthrene was needed to insure a high incidence of tumors if the skin were left in place. In experiment 1, mice were painted for 6 to 12 weeks. In the remainder of the experiments, painting was continued for 12 weeks, with the exception of experiment 7 in which the mice were painted for 10 weeks. After the prescribed period, painting was stopped for 7 to 14 days to allow for elimination of residual 3-methylcholanthrene. This was checked before operation by examination of the skin for fluorescence under an ultraviolet light.

A transverse incision was made across the base of the ear extending to the depth of the cartilage. The distal edge of the incision was grasped with forceps and the skin stripped from the underlying cartilage. When used as full-thickness transplants, the excised skin was appropriately divided into small pieces, each of which was folded so that the connective-tissue corium was on the outside and the epithelium on the inside. Each transplant was then rinsed in Ringer's solution containing 100 units of penicillin and 100 μg of streptomycin per ml before implantation. Pure epidermal transplants and transplants of connective-tissue corium were prepared by partial digestion of pieces of full-thickness skin in sterile, buffered trypsin solution. After digestion, the epithelium was

[5] Animal Foundation Laboratory Diet, manufactured by Standard Brands, New York City.

easily separated from its underlying connective tissue by methods previously described by Billingham and Medawar (*3*) and modified in this laboratory (*2*).

Only transplants of full-thickness skin were made from carcinogen-treated areas of the back. An ellipse of skin containing the painted area, with a surrounding margin of unpainted skin, was excised. The incision in the back was closed with sutures. The painted skin was appropriately divided and folded as described.

Transplants were placed subcutaneously in the back, axilla, or abdominal cavity according to the requirements of the individual experiments. Painted skin was also transplanted subcutaneously to mice of the same strains that had not been exposed to 3-methylcholanthrene.

Mice were examined 3 times a week for the presence of tumors. Some were killed because of ulcerated tumors of the skin, others at appropriately selected intervals of time.

At autopsy, all tissue blocks were fixed in Helly's solution. All transplants, regardless of size or apparent viability, were serially sectioned. From different levels in the tissue blocks, 8 to 15 slides were prepared. Sections were stained with hematoxylin and eosin.

OBSERVATIONS

Experiment 1

The ears of 90 strain A mice were painted with 3-methylcholanthrene for 6 to 12 weeks. The animals were divided into 3 groups of 30 each. The painted skin of the ears was excised and transplanted subcutaneously to the back as full-thickness skin (group I), as pure epithelium after partial digestion with trypsin and subsequent separation (group II), or corium (group III). Sixty-three mice survived the experimental period. The results are presented in table 1.

TABLE 1.—Autologous subcutaneous dorsal transplants of ear skin and component parts after 6 to 12 weeks of carcinogen treatment in strain A mice

Type of transplant	Full-thickness skin	Pure epidermis	Corium freed of epidermis	Total
Mice surviving experiment	19	25	19	63
Mice developing tumors	4	7	2	13 (21%)
Transplants performed	38	50	32	120
Transplants found	37	32	32	101
Transplants containing viable epithelium	31	13	26	70
Transplants containing tumor	0	0	0	0

The painting with 3-methylcholanthrene was effective in producing tumors (figs. 1, 4, 5, and 6). Of the 63 mice, 13 (21%) developed tumors

in painted areas of the ear or neck that remained after excision of the distal ear skin for transplantation.

Of 120 internal transplants performed, 101 were identified at autopsy. Seventy contained cysts lined by viable epithelium (fig. 7); none contained tumor. If tumors had developed in transplants with the frequency observed in the painted areas from which the transplanted skin had been taken, 14 tumors should have been detected in the 70 viable transplants studied. The failure of tumor development could not be attributed to necrosis of the transplants (31 of 38 full-thickness skin transplants contained viable epithelium). The failure of tumor formation after transplantation focused attention on this aspect rather than on the initial objective of comparing pure epidermal transplants with those of full-thickness skin transplants.

Experiment 2

The ears of 72 strain A mice were painted with 3-methylcholanthrene for 12 weeks. In 36 of these mice full-thickness skin from the painted area was transplanted to the subcutaneous tissue of the back. In the remaining 36 mice pure epidermis was similarly transplanted. At 2-week intervals, from 8 to 20 weeks after operation, the animals were killed and autopsies performed.

The interval between operation and autopsy did not influence the incidence of tumors of the skin in the painted areas adjoining those from which the transplants had been taken. The longer the interval, however, the smaller the number of pure epidermal transplants surviving. This observation is in accordance with studies of epidermal transplants of unpainted skin (*2*). The results are presented in table 2.

TABLE 2.—Autologous subcutaneous dorsal transplants of skin or pure epidermis after 12 weeks of carcinogen treatment in strain A mice

Type of transplant	Full-thickness skin	Pure epidermis	Total
Mice surviving experiment	32	28	60
Mice developing tumors	18	24	42 (70%)
Transplants performed	64	28	92
Transplants found	49	22	71
Transplants containing viable epithelium	37	7	44
Transplants containing tumor	1	0	1

Of 60 animals surviving the experimental period, 42 (70%) developed tumors in the painted areas from which transplants were taken. Of the 71 transplants made from these areas before tumor developed, 44 (62%) contained viable epithelium; but, with one exception, none contained tumors.

Experiment 3

The ears of 49 mice (nearly equally divided among strains C57BL, A, C3H, and BALB/c) were painted with 3-methylcholanthrene for 12 weeks. The painted skin of the ears was excised and divided. Half of this full-thickness skin was transplanted in two equal parts into the axillas of the painted mouse. The other half of the skin was transplanted to the subcutaneous tissues of the back of an unpainted mouse of the same strain. Mice were killed between 12 and 20 weeks after operation. The results are summarized in table 3.

TABLE 3.—Autologous subcutaneous axillary transplants and isologous subcutaneous dorsal transplants of ear skin after 12 weeks of carcinogen painting

	Strain of mice	C57BL	A	C3H	BALB/c	Total
A. Painted mice receiving autologous transplants	1. Mice surviving experiment	8	12	8	9	37
	2. Mice developing tumors	3	9	3	3	18 (49%)
	3. Transplants performed	16	24	16	18	74
	4. Transplants containing viable epithelium	16	21	16	15	68
	5. Transplants containing tumor	0	0	0	1	1
B. Unpainted mice receiving isologous transplants from painted donors	1. Mice surviving	11	3	3	9	26
	2. Transplants performed	22	6	6	18	52
	3. Transplants containing viable epithelium	22	6	6	9	43
	4. Transplants containing tumor	0	0	0	0	0

Thirty-seven painted mice survived the experimental period. Eighteen developed skin tumors of the ear in the painted area. Of the 74 transplants of painted skin made to the axillas of the same mice, 68 (92%) contained surviving epithelium. Of 52 transplants made to the back of unpainted recipients, 43 (83%) contained viable epithelium. With the exception of a papilloma found in one autologous axillary transplant, no tumors were found.

Experiment 4

The ears of 44 mice of the 4 different strains were painted with 3-methylcholanthrene for 12 weeks. The painted skin was excised and divided, and both halves were transplanted into the abdominal cavity of the painted individual. The mice were killed between 12 and 30 weeks after operation. The results are shown in table 4. Nine (29%) of 31 mice surviving developed tumors in the remaining portions of the painted area. Of the 62 transplants in the surviving animals, 59 (95%) contained viable epithelium, yet only 2 of these contained tumor.

TABLE 4.—Autologous abdominal transplants of ear skin after 12 weeks of carcinogen painting

Strain of mice	C57BL	A	C3H	BALB/c	Total
Mice surviving experiment	5	9	8	9	31
Mice developing tumor	0	2	4	3	9 (29%)
Transplants performed	10	18	16	18	62
Transplants found	9	18	15	17	59
Transplants containing viable epithelium	9	18	15	17	59
Transplants containing tumor	0	2	0	0	2 (3%)

Experiment 5

The backs of 48 mice (12 each of strains C57BL, A, C3H, and BALB/c) were painted for 12 weeks. The painted animals were divided into 3 groups. The painted skin was excised, and half was transplanted subcutaneously to the back (group I), the axilla (group II), or the abdominal cavity (group III). The remaining half was transplanted subcutaneously to the back of an unpainted mouse of the same strain. The results are summarized in table 5. Of the 34 mice surviving the experimental period, 9 (26%) had had tumors at the time of these operations, and 16 (47%) more had developed tumors in the painted area by the time of death. Eighteen of 19 similarly painted control animals had developed tumors in 20 weeks. Mice were killed at intervals between 12 and 38 weeks after transplantations.

TABLE 5.—Autologous transplants to different internal sites and isologous subcutaneous dorsal transplants of dorsal skin after 12 weeks of carcinogen painting

	Site of autologous internal transplant	Back	Axilla	Abdomen	Total
A. Painted mice receiving autologous transplant	1. Mice surviving experiment	13	13	8	34
	2. Mice with tumors at time of operation	4	4	1	9 (26%)
	3. Total mice with tumors by time of death	6	4	6	16 (47%)
	4. Transplants performed	26	26	16	68
	5. Transplants containing viable epithelium	18	23	12	53
	6. Transplants containing tumor	4	6	4	14 (26%)
B. Unpainted mice receiving isologous transplants from painted donors	1. Transplants performed in surviving mice	28	22	20	70
	2. Transplants containing viable epithelium	14	14	12	40
	3. Transplants containing tumor	3	0	4	7 (18%)

Similar results were obtained with different strains and transplantation sites. Of the 53 transplants containing viable epithelium, 14 (26%) contained tumor (figs. 8, 9, and 10). This incidence is nearly identical with the incidence of tumors at the time of operation and suggests that tumor development did not occur after transplantation. Tumors were found in transplants only if they existed prior to transplantation. Similarly, the incidence of tumors in the transplants to unpainted recipients (18%, table 5, line B3) was even lower than the incidence of tumors in the painted areas from which these transplants were taken (26%, table 5, line A2).

Experiment 6

The backs of 40 mice of strain C3H were painted with 3-methylcholanthrene for 12 weeks. The painted areas from 10 mice were excised at each of the following intervals after painting: 2, 5, 8, and 12 weeks. The excised skin was divided and each half was transplanted to the axillas. Mice were killed 20 weeks after painting. The results are shown in table 6.

TABLE 6.—Autologous axillary transplants of dorsal skin after 12 weeks of carcinogenic painting and varying intervals between painting and transplantation

Weeks between painting and transplantation	2	5	8	12
Mice painted	10	10	10	10
Mice surviving experiment	4	2	8	7
Tumors of back at operation and autopsy	1	0	1	0
Transplants performed	8	4	16	14
Transplants containing viable epithelium	8	4	14	14
Transplants containing tumor	1	1	5	3

The incidence of tumors was low. When the skin was transplanted less than 8 weeks after painting, there were 12 viable transplants, but only 2 (17%) contained tumor. When the skin was transplanted 8 weeks or longer after painting, there were 28 transplants containing viable epithelium of which 8 (29%) contained tumor. The slightly increased incidence of tumors after the longer interval between painting and transplantation suggested that further studies be directed toward determining whether or not tumor formation occurred only before transplantation and not thereafter.

Experiment 7

The backs of 39 mice of strain C3H were painted for 10 weeks. Nine of these were kept as controls. Of the 8 controls that survived the experimental period 7 (88%) developed malignant tumors in the painted area. In the 30 experimental animals the painted areas were excised. Nearly all contained grossly identifiable tumor. Two fragments from

the tumors were transplanted to the axillas of each animal. Histologic sections prepared from the rest of the excised skin revealed tumors in 25 of the 26 animals that survived the experimental period. Many had multiple tumors. These were classified according to the most aggressive of the tumors identified. Thirteen (50%) were invasive carcinomas or sarcomas. Mice were killed between 6 and 14 weeks after operation. The results are presented in table 7.

TABLE 7.—Autologous axillary transplants of dorsal skin containing carcinogen-induced tumors

Mice painted	30
Mice surviving experiment	26
Mice with papillomas when transplanted	12 (46%)
Mice with malignant tumors when transplanted	13 (50%)
Transplants performed	52
Transplants containing viable epithelium	42
Transplants containing papillomas	4 (10%)
Transplants containing malignant tumors	17 (40%)
Total number of tumors in transplants	21 (50%)

Of the 42 viable transplants, 4 (10%) contained papillomas and 17 (40%) contained malignant tumors. The incidence of malignant tumors in the original skin site and in the transplants was very similar. The incidence of benign papillomas in the transplants was lower than in the original site. This suggested that some papillomas regressed.

Experiment 8

The backs of 22 mice (strains C57BL, A, C3H, and BALB/c) were painted for 12 weeks. All the skin from a square area on the back approximately 2 × 2 cm was excised, except for a central portion (4 × 4 mm) which was left in place with its blood supply intact (fig. 2). A skin flap was developed by extending the two transverse incisions laterally on one side (fig. 2). This flap was elevated, pulled across the midline and sutured in place, thus covering the undisturbed painted area (fig. 3). The results are summarized in table 8.

TABLE 8.—Tumor incidence in dorsal skin covered by a skin flap after 12 weeks of carcinogen painting

Strain of mice	C57BL	A	C3H	BALB/c	Total
Mice operated	6	6	6	4	22
Mice surviving	3	6	5	3	17
Mice developing external skin tumor	1	2	0	1	4 (24%)
Mice with surviving epithelium in buried skin	1	4	5	1	11
Tumor present in buried skin	1	4	2	0	7 (64%)

Seventeen mice survived and were killed between 12 and 22 weeks after operation. Of these, 4 (24%) developed tumors in the unresected surface skin of the back. The low tumor incidence in the surface skin is presumed the result of the nearly complete excision of the painted area. In 11, the buried skin had developed into cysts lined by viable epithelium. Seven (64%) of these cysts contained tumors. Thus a high incidence of tumors was found in buried fragments of skin when such fragments were buried with their blood supply intact, instead of being freely transplanted as in the experiments described.

DISCUSSION

In this study segments of pure epithelium and full-thickness skin from areas painted with 3-methylcholanthrene were transplanted successfully to subcutaneous and intraperitoneal sites. No tumors developed in the transplants of pure epithelium. Few tumors developed in the transplants of full-thickness skin unless they were already present in the skin before transplantation. Tumors did develop in the painted areas from which the transplants had been taken.

Benign papillomas present in the transplanted skin at the time of transplantation regressed after transplantation. Malignant tumors present in the transplanted skin progressed after transplantation. It was concluded, therefore, that some factor incident to transplantation inhibits the development of malignant tumors and causes benign tumors to regress.

Similar observations have been reported by others. Kreyberg (*4*) in 1925 found that benign papillomas of the skin induced with tar regressed when transplanted internally, whereas fully developed carcinomas continued to grow. Cornman (*5*) observed that tumors failed to develop in transplants of skin exposed to an effective dose of carcinogen. Billingham, Orr, and Woodhouse (*6*) failed to obtain tumors in transplants of both pure epithelium and "thin Thiersch grafts" previously treated with 3-methylcholanthrene. Their transplants were placed on surface sites where later identification was less certain than with transplants placed in the subcutaneous tissues. They obtained a few tumors in similarly transplanted "thick Thiersch grafts." The tumors appeared relatively soon after transplantation. The authors suggested that the tumors may already have been present in the skin when grafted. Their results were similar to those herein reported. Furthermore, in experiments 5 and 6, careful search of the skin before transplantation did reveal tumors in nearly the same proportion as was later found in the transplants.

Rous and Kidd (*7*) have shown that skin treated with carcinogen and left in place is permanently altered. Even though no tumor is visible, a tumor can be produced in the painted area by a nonspecific promoting agent. Tumor induction may thus be divided into two stages: initiation and promotion. The present study does not indicate whether transplantation inhibits tumor development by a reversal of the initiating process or by an inhibition of the promoting factors. Studies by Cowen (*8*) suggest

that transplantation does not reverse the initiating process. Skin was treated with 7,12-dimethylbenz[*a*]anthracene, excised, transplanted to an unpainted mouse of the same strain, and then painted with promoting agent (croton oil). Benign papillomas developed in the transplants of skin previously treated with carcinogen and not in transplants of skin without previous treatment with carcinogen.

Studies of tumor development in transplants of tissue exposed to carcinogen focus attention on these important initiating and promoting factors and, therefore, have advantages over studies of transplants of fully developed tumors. These are concerned only with those factors necessary for continued survival and growth of a previously induced tumor.

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PLATES

PLATE 212

FIGURE 1.—Mouse of strain A, painted with 3-methylcholanthrene for 12 weeks. Skin of ears excised and transplanted subcutaneously to back. Tumors which subsequently appeared in residual skin of ears are shown. No tumors developed in transplants.

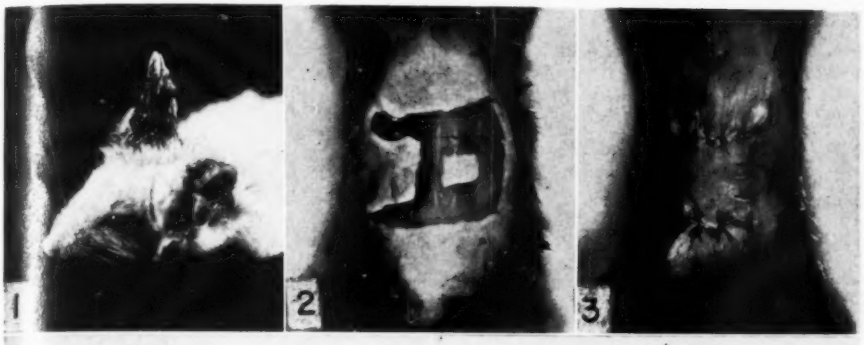
FIGURE 2.—Mouse from experiment 8. Painted skin was excised except for central area measuring 4 × 4 mm. Lateral incisions were made to *left* to create skin flap.

FIGURE 3.—Mouse from experiment 8. Skin flap shown in figure 2 was pulled across midline, covering 4 × 4 segment of painted skin, and held in place with interrupted sutures.

FIGURE 4.—Papilloma of mouse painted with 3-methylcholanthrene and killed 8 weeks after painting. × 29

FIGURE 5.—Invasive and ulcerated carcinoma of skin of mouse painted with 3-methylcholanthrene and killed 8 weeks after painting. × 28

FIGURE 6.—Higher magnification of same tumor shown in figure 5. Small sheets of squamous carcinoma are present among muscle bundles of panniculus carnosus. × 450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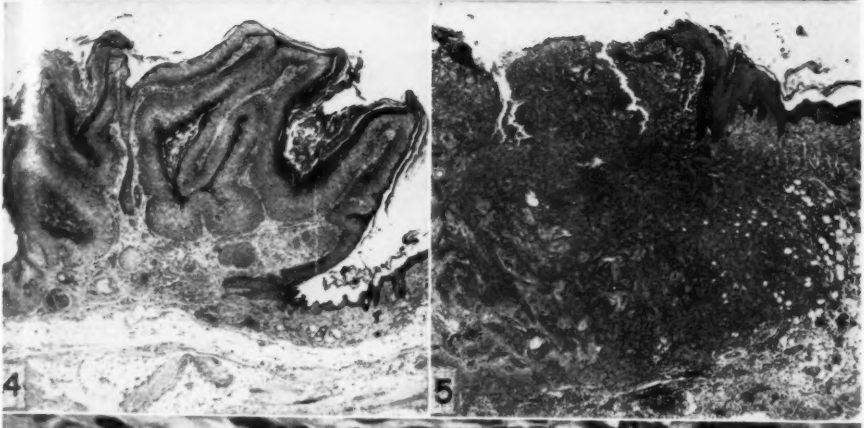
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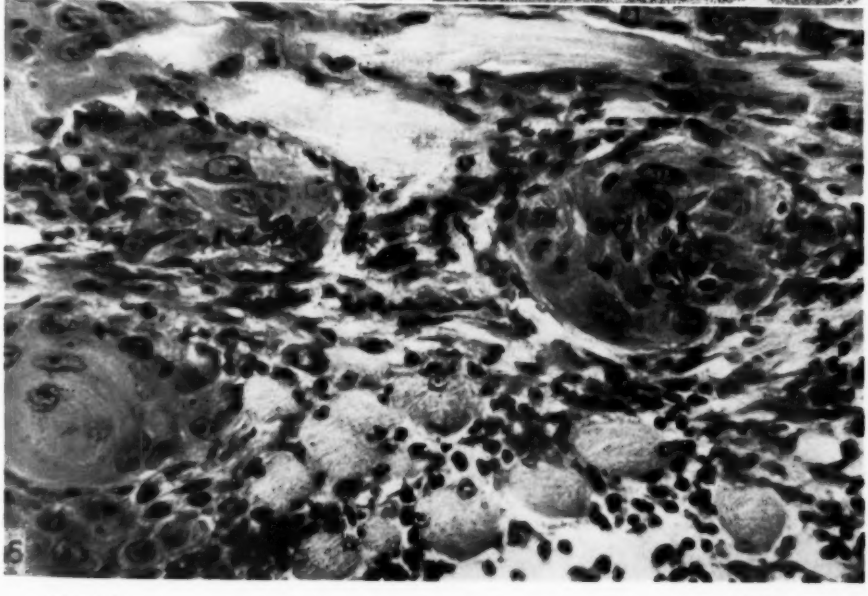
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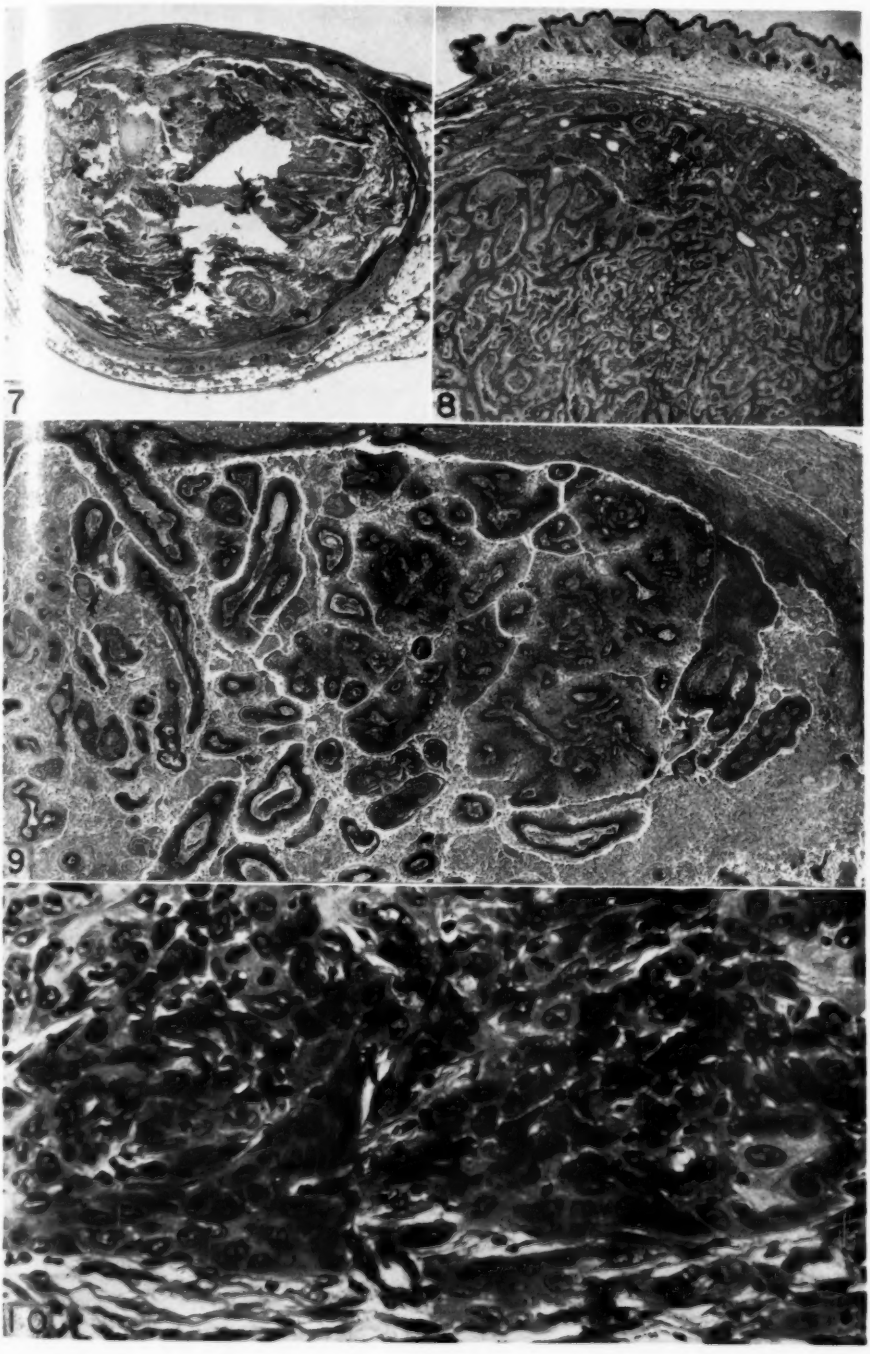
PLATE 213

FIGURE 7.—Subcutaneous transplant of skin of mouse previously painted with 3-methylcholanthrene. Papilloma similar to that shown in figure 4 was present in skin used for transplant. Cyst lined by well-oriented stratified squamous epithelium and filled with desquamated cells. × 29

FIGURE 8.—Subcutaneous transplant of skin of mouse previously painted with 3-methylcholanthrene. Papilloma present in skin used for transplant. Cyst filled with well-differentiated squamous papilloma. × 28

FIGURE 9.—Subcutaneous transplant in same mouse shown in figures 4 and 5. Skin previously painted with 3-methylcholanthrene, and contained invasive carcinoma when transplanted. Transplant contains large papilloma. In ulcerated area to *right* it also contains an invasive carcinoma. See figure 10. × 19

FIGURE 10.—Higher magnification of ulcerated area shown on *right* of figure 9. Sheet of squamous carcinoma containing numerous mitoses is shown. × 369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Carcinogenic Activity of Analogues of *p*-Dimethylaminoazobenzene. IV. Activity of the Quinoline Analogues [1, 2]

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SUMMARY

Thirteen isomers of the *p*-dimethylaminophenylazoquinolines and their corresponding N-oxides were investigated for hepatic carcinogenic activity in Sprague-Dawley rats. The dyes were all fed at the 0.03 percent level, and the more active isomers were also examined at the 0.01 percent level. The isomers are listed in order of decreasing activity, with semiquantitative values based on *p*-dimethylaminoazobenzene as 6: 6-(*p*-dimethylaminophenylazo)quinoline, 200+; 5-(*p*-dimethylaminophenylazo)quinoline-1-oxide, 200+; 5-(*p*-dimethylaminophenylazo)quinoline, 150; 6-(*p*-dimethylaminophenylazo)-quinoline-1-oxide, 100; 4-(*p*-dimethylaminophenylazo)quinoline-1-oxide, 22; 4-(*p*-dimethylaminophenylazo)-quinoline, 15; 3-(*p*-dimethylaminophenylazo)quinoline-1-oxide, 3-(*p*-dimethylaminophenylazo)quinoline, 7-(*p*-dimethylaminophenylazo)quinoline-1-oxide, 7-(*p*-dimethylaminophenylazo)quinoline, 8-(*p*-dimethylaminophenylazo)quinoline-1-oxide, 8-(*p*-dimethylaminophenylazo)quinoline, and 2-(*p*-dimethylaminophenylazo) quinoline-1-oxide, with all the latter less than 2.—J. Nat. Cancer Inst. 26: 1461-1464, 1961.

IN PREVIOUS reports (*1*, *2*), we have shown that the unsubstituted ring of *p*-dimethylaminoazobenzene (DAB) can be replaced by pyridine and pyridine N-oxide and thus we have obtained a number of compounds with varying degrees of carcinogenic activity. The interesting results observed in the pyridine series led us to investigate the isomeric *p*-dimethylaminophenylazoquinolines and their corresponding N-oxides. In this new series, we have the interesting possibility of attaching the azo linkage to either the pyridine or benzene rings of the quinoline nucleus and thereby preparing compounds which can be considered pyrido analogues of DAB or benzo analogues of the previously prepared pyridine

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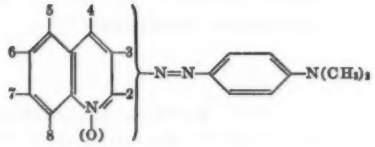
azo compounds. A general formula representing all the compounds prepared is:



The seven position isomers of the *p*-dimethylaminophenylazoquinolines and their corresponding N-oxides were prepared (*3*) and coded Q2 through Q8 and QO2 through QO8, respectively, depending on the position of attachment of the *p*-dimethylaminophenylazo group to the quinoline nucleus. All except Q2 were then fed to rats at the 0.03 percent level, and the more active compounds were also fed at the 0.01 percent level. In addition to determining tumor incidences, it was possible by the use of the formula of Miller and Miller (*4*) to assign semiquantitative carcinogenic activities to these new compounds.

Young male rats of the Sprague-Dawley strain, approximately 8 weeks of age and weighing 150 to 200 g, were distributed as equally as possible in initial body weight into groups of 10 animals each. Each group was fed a diet, patterned after the "low protein, low riboflavine" diet of Miller *et al.* (*5*), to which had been added one of the azo compounds at a level of 0.03 percent. The composition of the basal diet on a kg basis was as follows: crude casein, 120 g; cerelose, 770 g; Osborne and Mendel salt mixture, 40 g; corn oil, 50 g; Vitab (rice bran concentrate, obtained from Charles Bowman Co.), 20 g; riboflavine, 0.5 mg; vitamin A palmitate, 67,500 I.U., for which we are grateful to Charles Pfizer and Co., Inc., for a generous supply.

In each experiment, groups received DAB at the 0.06 percent as well as at the 0.03 percent level. The control group received only the basal diet. All the rats were kept individually in screen-bottomed cages and were offered food and water *ad libitum*. Laparotomies were performed at the indicated times and microscopic examinations[4] were made whenever an animal died or at the end of the experiment.

The tumor incidences for the rats fed the various quinoline analogues of DAB at the 0.03 percent level are shown in table 1. Data for DAB and 3′-Me-DAB at the 0.03 percent level are included. Since the levels of activity of several compounds were indistinguishable at the 0.03 percent level, these were examined at the 0.01 percent level and are recorded in table 2. With the use of the Miller formula (*4*) the values for the quinoline series of compounds were calculated, with the value of 6 for DAB. Since only limiting values were obtained for several compounds, the 0.01 percent values were then used. These data are illustrated in table 3.

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[4] We with to express appreciation to Dr. George T. Hoffman of St. Vincent's Hospital, New York City, for the microscopic evaluation of the tumors.

Even at this lower level of feeding it was possible to obtain only limiting values with QO5 and Q6.

TABLE 1.—Carcinogenicities of quinoline dyes in relation to DAB*

Code	Incidence of liver tumors†	
	4 Months	6 Months
Control (no dye)	0/10	0/10
DAB‡ (0.06%)	7/10	9/10
DAB	0/10	5/10
3'-Me-DAB§	5/11	10/11
QO2‖	0/10	0/10
Q3¶	0/10	1/10
QO3	0/10	0/10
Q4	5/10	9/9
QO4	8/10	10/10
Q5	9/9	—
QO5	10/10	—
Q6	9/9	—
QO6	9/10	10/10
Q7	0/10	0/10
QO7	0/10	0/10
Q8	0/10	0/10
QO8	0/10	0/10

*Level in diet, 0.03 percent unless noted.

†Number of rats with tumors/number of rats in experiment.

‡*p*-Dimethlyaminoazobenzene; *Chemical Abstracts'* nomenclature: *N,N*-dimethyl-*p*-phenylazoaniline.

§3-Methyl-*p*-dimethylaminoazobenzene; *Chemical Abstracts'* nomenclature: *N,N*-dimethyl-*p*-(*m*-tolylazo) aniline.

‖Typical common name in this series: 2-(*p*-dimethylaminophenylazo)quinoline-1-oxide; *Chemical Abstracts'* nomenclature: *N,N*-dimethyl-*p*-(2-[quinolyl-1-oxide]azo)aniline.

¶Typical common name in this series: 3-(*p*-dimethylaminophenylazo)quinoline; *Chemical Abstracts'* nomenclature: *N,N*-dimethyl-*p*-(3-quinolylazo)aniline.

TABLE 2.—Comparison of the carcinogenicities of quinoline dyes*

Code	Incidence of liver tumors† (4 months)
Q5	8/9
QO5	10/10
Q6	10/10
QO6	5/10

*Level in diet, 0.01 percent.

†Number of rats with tumors/number of rats in experiment.

DISCUSSION

As might have been anticipated from the results obtained in the pyridine series (*1*), QO4 is the most active of the compounds substituted on the pyridine side of the quinoline nucleus. However, the high activity of the 5 and 6 substituted compounds is quite surprising and in contrast to the lack of activity in the 7 and 8 substituted compounds. Q6 is more active

TABLE 3.—"Relative Reactivity"* of quinoline dyes in relation to DAB†

Code	0.03 Percent	Code	0.01 Percent
DAB	= 6	Q5	= 150
Q3	= 2−‡	QO5	= 200+
QO3	= 2−	Q6	= 200+
Q4	= 15	QO6	= 100
QO4	= 22		
Q5	= 40+§		
QO5	= 50+		
Q6	= 40+		
QO6	= 40+		

*Relative reactivity $= \frac{6 \times \text{months fed} \times \text{percent tumors of test compound}}{\text{months fed} \times \text{percent tumors of DAB}}$.

See reference 4 where equimolar levels of dyes are compared. Our use of equal percentage levels will give activities lower than those that would be obtained with equimolar levels.

†Level in diet 0.03 and 0.01 percent.

‡Below 2 in activity and is inactive in comparison to DAB. A higher level of feeding might give a more accurate comparison.

§Shows a minimum activity, with calculation given in asterisk footnote. Lower levels of feeding would be necessary to indicate a more exact value. This is further illustrated in the 0.01 percent column where minimum value of 200 is sometimes obtained.

than its N-oxide, QO6, which has not been the usual situation. Levels of feeding below 0.01 percent will be necessary to decide whether QO5 or Q6 is the most active hepatic azo carcinogen so far discovered.

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Announcements

VIII International Cancer Congress

The VIII International Cancer Congress will take place July 22 to 28, 1962, in Moscow, under the auspices of the International Union Against Cancer.

Twenty-two cancer topics have been selected for the program:

> Role of viruses in the origin of cancer; precancerous lesions; biochemistry of cancer; immunology and genetics of tumors; tumor-host relationships and hormone status; experimental research and clinical oncology; radiobiology; epidemiological studies on cancer; occupational cancer; etiology and pathogenesis of liver cancer; cancer detection; radical cancer surgery; biological approach to cancer surgery; new methods of cancer therapy; new methods in radiotherapy; carcinoma of the stomach; carcinoma of the lung; carcinoma *in situ* of the cervix; long-term results in therapy of cancer; care of patients with advanced cancer; cancer control; miscellaneous.

Applications for reading papers at the Congress will be considered on condition that both the application and abstract of the paper, not exceeding 250 words, are submitted not later than November 1, 1961.

The registration fee is 30 U.S. dollars per member, if sent before April 1, 1962.

All foreign members of the Congress will be serviced by the Soviet travel agency "Intourist" which offers them facilities in several price categories.

All the information concerning the Congress, as well as enrollment forms and applications for reading papers and showing films may be obtained through:

Prof. L. M. Shabad, General Secretary
USSR National Organizing Committee
VIII International Cancer Congress
Academy of Medical Sciences of the USSR
14 Solyanka
Moscow, USSR

or

Dr. Harold F. Dorn, General Secretary
International Union Against Cancer
National Institutes of Health
Bethesda 14, Maryland

Travel allotments will be available to a limited number of selected scientists and physicians residing in the USA. The grant will cover round-trip economy jet fares, per diem allowance, and registration fee.

Further information regarding the allotments can be obtained from the Chairman, USA National Committee on the International Union Against Cancer, 2101 Constitution Ave. NW., Washington 25, D.C.

Manuscripts Approved for JNCI, April 15 to May 15, 1961

"Thresholds in linear dose-response models for carcinogenesis," by Nathan Mantel, Walter E. Heston, and Joan M. Gurian.

"A hepatitis virus complicating studies with mouse leukemia," by Robert A. Manaker, Chester V. Piczak, Alice A. Miller, and Mearl F. Stanton.

"Aminofluorenols as urinary metabolites of *N*-2-fluorenylacetamide," by J. H. Weisburger, E. K. Weisburger, and H. P. Morris.

"On the *in vitro* and *in vivo* protein-binding on *N*-2-fluorenylacetamide and related compounds," by J. H. Weisburger, P. H. Grantham, H. P. Morris, and E. K. Weisburger.

"Microspectrophotometric determinations of DNA in primary and metastatic mouse mammary tumors," by Elizabeth W. Chu and Richard A. Malmgren.

"Mammary carcinogenesis by 3-methylcholanthrene. III. Induction of mammary carcinoma and milk secretion in male rats bearing ovarian grafts," by Thomas L. Dao and Mary J. Greiner.

"Uterine cancer in Connecticut: Late deaths among five-year survivors," by John C. Bailar.

"Cytophotometric data in fluorochromed cervical cells," by Marianna Masin and Francis Masin.

"Comparisons between the survival of grafted skin, ovaries, and tumors in mice across histocompatibility barriers of different strength," by Owe E. A. Linder.

"Tolerance induced by parity in mice incompatible at the *H*-2 locus," by Edward Breyere and Morris K. Barrett.

"Genetic and environmental influences on teratocarcinogenesis in mice," by L. C. Stevens and Judith A. Mackensen.

"Studies on suppression of homograft response with folic-acid antagonists," by Stewart R. Humphreys, Michael A. Chirigos, Kenneth L. Milstead, and Abraham Goldin.

"Induction of epidermoid carcinoma in lungs of rats: A 'new' method related to pulmonary infarction," by Mearl F. Stanton and Robert Blackwell.

"Smoking habits of men and women," by E. Cuyler Hammond and Lawrence Garfinkel.

" 'Safety' testing of carcinogenic agents," by Nathan Mantel and W. Ray Bryan.

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